Digital PCR to Detect and Quantify Heteroresistance in Drug Resistant *Mycobacterium tuberculosis*

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**Abstract**

Drug resistance in *Mycobacterium tuberculosis* presents an enormous public health threat. It is typically defined as >1% of drug resistant colonies using the agar proportion method. Detecting small numbers of drug resistant TB in a population, also known as heteroresistance, is challenging with current methodologies. Here we have utilized digital PCR to detect heteroresistance within *M. tuberculosis* populations with excellent accuracy versus the agar proportion method. We designed dual TaqMan-MGB probes to detect wild-type and mutant sequences of *katG* (315), *rpoB* (531), *gyrA* (94,95) and *rrs* (1401), genes that associate with resistance to isoniazid, rifampin, fluoroquinolone, and aminoglycoside respectively. We generated heteroresistance mixtures of susceptible and extensively drug resistant TB, followed by DNA extraction and digital PCR. Digital PCR yielded a close approximation to agar proportion's percentages of resistant colonies, and yielded 100% concordance with agar proportion's susceptible/resistant results. Indeed, the digital PCR method was able to identify mutant sequence in mixtures containing as little as 1000:1 susceptible:resistant TB. By contrast, real-time PCR or PCR followed by Sanger sequencing were less sensitive and had little resolution to detect heteroresistance, requiring fully 1:1 or 10:1 susceptible:resistant ratios in order to detect resistance. Our assay can also work in sputum so long as sufficient quantities of TB are present (>1000 cfu/ml). This work demonstrates the utility of digital PCR to detect and quantify heteroresistance in drug resistant TB, which may be useful to inform treatment decisions faster than agar proportion.

**Introduction**

Multidrug-resistant tuberculosis (MDR-TB), defined as resistance of >1% of colonies to isoniazid (INH) and rifampin (RIF), was found in 3.3% of new cases and 21% of recurrent TB cases globally [1], and many strains harbor extensive resistance to additional drugs. Some TB patients harbor mixed populations of drug susceptible and resistant organisms, a phenomenon which is referred to as heteroresistance [2].

Heteroresistance allows subpopulations of bacteria to grow despite the presence of antibiotics and is thus a precursor to full resistance [3]. The phenomenon is not rare in *M. tuberculosis* and has been reported for isoniazid, rifampin, ethambutol, streptomycin, and fluoroquinolones [4–6]. It can be detected by culture-based methods (i.e., when more than 1 but less than 100% of colonies are resistant to drug), or by simultaneous detection of wild-type and mutated sequences using PCR-based techniques, such as restriction fragment length polymorphism [4], sequencing [7] or line probe assays [6,8].

Most of the latter PCR-based methods are laborious and technically difficult. Moreover, detection of low amounts of mutant template is challenging with PCR since the abundant (in this case wild-type) sequence may preferentially amplify. This possibility urges caution given the proliferation of PCR-based methods to detect MDR TB in the field. For example, it has been reported that between 65% and 100% of *rpoB* mutant DNA is required to be detected by the Xpert MTB/RIF assay [9]. Likewise, the line probe based Genotype MTBDR assay can detect heteroresistance of MTB but requires that the relative proportion of resistant organisms is ≥10% [8]. Recently, a molecular beacon assay was developed to detect mutant targets of *gyrA* gene, but again required the presence of 5–10% of mutant DNA to be detected in a mixture with wild type DNA [5]. Importantly, if detected, none of these assays yield a precise measure of how much heteroresistance exists.

In 1999 the term digital PCR was used to describe dilution of DNA to single copies to improve the sensitivity of PCR to detect rare cancer-associated mutations amidst an excess of wild-type sequence [10]. Here we have investigated the role of digital PCR to detect and quantify heteroresistance within mixed TB populations.

**Materials and Methods**

Mycobacterial strains and culture conditions

Mycobacterial strains used in this study included *M. tuberculosis* H37Rv (ATCC 2794) and a clinical extensively drug resistant (XDR) TB isolate (100% resistant to isoniazid, rifampin, ofloxacin, amikacin, kanamycin, streptomycin by agar proportion method, with the *katG315, rpoB531, gyrA94,95, and rrs1401 mutations* from the Mycobacteriology Service Unit, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bang-
kok, Thailand. All work was approved by the University of Virginia Institutional Biosafety Committee and Human Investigation Committees. Tb isolates were cultured on Löwenstein-Jensen medium at 37°C for two weeks. Cell suspensions were prepared in Middlebrook 7H9 (7H9 broth supplemented with Middlebrook OADC enrichment (Difco, Livonia, MI, USA) and adjusted to 0.5 McFarland.

H37Rv:XDR-TB mixtures

The 0.5 McFarland XDR-TB was diluted 10-fold serially in M7H9 plus OADC. One ml of each XDR-TB suspension (undiluted, 1/10, 1/100, 1/1000) was mixed with 1 ml of 0.5 McFarland H37Rv to construct mixtures of 1:1, 10:1, 100:1, and 1000:1 respectively.

DNA extraction

DNA was isolated from each TB mixture. Briefly, 1 ml of 0.5 McFarland H37Rv, XDR-TB and each H37Rv:XDR-TB mixtures were transferred to 2 ml screw cap tube, centrifuged at 21,000 x g for 10 min, and the pellets were re-suspended in 1 ml Tris-EDTA (TE) buffer, boiled at 100°C for 3 min, and supernatants were stored at −20°C as DNA template.

Oligonucleotides

Primers and probes for katG, rpoB, gyrA, and rrs were sourced based on sequences of H37Rv and the XDR-TB isolate. The rpoB primers and the mutant probe were those reported by Espasa et al [11], while the primers and probes of katG were those of Yesilkaya et al [12]. The primers and probes of gyrA, rrs, and the rpoB wild-type probe were designed for this work using Primer Express3 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). All sequences are shown in Table 1.

**Table 1.** Primer and probe sequences.

| Gene  | Primers and Probes | Sequences                          |
|-------|--------------------|------------------------------------|
| katG  | katG-F: 5’-TGCTCCGTGAGGACAGAT-3’ |                                    |
| katG-R: 5’-TCGAGGAAACTGTTGCTCCATT-3’ |                                    |
| katG315-wt: 5’-FAM-CGATGCGGCTGG-MGB-3’ |                                    |
| katG315-mt: 5’-VIC-CGATGCGGCTGG-MGB-3’ |                                    |
| rpoB  | rpoB-F: 5’-ACGCGACAGGTGATCAACAT-3’ |                                    |
| rpoB-R: 5’-GGCCACCTGACAGG-3’ |                                    |
| rpoB531-wt: 5’-TexasRed-CCCCAGCGCCACATCGTCCG-3’ |                                    |
| rpoB531-mt: 5’-Cy5-CAGCGCCACATCGTCCG-3’ |                                    |
| gyrA  | gyrA-F: 5’-AGACCATGGGCAACTACAC-3’ |                                    |
| gyrA-R: 5’-TTTCTTACACAGTCTACAT-3’ |                                    |
| gyrA94,95-wt: 5’-FAM-GATCTACAGCAGGCTGG-MGB-3’ |                                    |
| gyrA94,95-mt: 5’-VIC-GATCTACAGCAGGCTGG-MGB-3’ |                                    |
| rrs   | rrs-F: 5’-AACCTTTAAAGCGCGTCCTA-3’ |                                    |
| rrs-R: 5’-CTTCCTCCGAGGTGTTAG-3’ |                                    |
| rrs1401-wt: 5’-FAM-GCCCGTACGTAC-MGB-3’ |                                    |
| rrs1401-mt: 5’-VIC-CCCCGTACGTAC-MGB-3’ |                                    |

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Real-Time PCR

PCR mixtures (10 μl) consisted of 5 μl of 2×Quantitect Multiplex NoROX PCR master mix (Qiagen Inc, Valencia, CA, USA), 0.1 μl of 50 μM of each forward and reverse primer except for katG (0.16 μl), 0.04 μl of 50 μM of each wild-type and mutant probe, 2.72 μl nuclease-free water except for katG (2.6 μl) and μl of DNA template. Real-time PCR of each gene was performed on a ViiA7 [Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA] with initial denaturation at 95°C for 10 min, followed by 45 cycles denaturation at 95°C for 15 sec, and annealing and extension at 63°C for 1 min.

Digital PCR

DNA from each H37Rv:XDR-TB mixture was diluted 10 fold serially in nuclease free water and subjected to real-time PCR for *rpoB* in 10 replicates in order to calculate the percentage of positive detections at each dilution. The 1/10000 dilution was chosen and used to perform digital PCR for each gene. Digital PCR mixtures (10 μl) consisted of 5 μl of 2×Quantitect Multiplex NoROX PCR master mix (Qiagen Inc, Valencia, CA, USA), 0.1 μl of 50 μM of each forward and reverse primer except for katG (0.16 μl), 0.04 μl of 50 μM wild type probe, 0.04 μl of 50 μM mutant probe, 2.72 μl nuclease-free water except for katG (2.6 μl) and 2 μl of diluted DNA template. Digital PCR was performed in 384 well formats on a ViiA7 [Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA] with initial denaturation at 95°C for 10 min, followed by 45 cycles (except for katG, 55) denaturation at 95°C for 15 sec, annealing and extension at 63°C for 1 min.

Digital PCR on TB in sputum

The 1:1, 10:1, 100:1, and 1000:1 H37Rv:XDR-TB mixtures were diluted 10 fold serially in nuclease free water and subjected to real-time PCR for *rpoB* in 10 replicates in order to calculate the percentage of positive detections at each dilution. The dilution that yielded approximately 50% PCR positive was chosen and for digital PCR.

Sequencing of loci

The *katG, rpoB, gyrA, and rrs* loci were amplified using the primers of Campbell et al [13]. Each 25 μl PCR mixture contained 12.5 μl HotStarTaq master mix (Qiagen Inc, Valencia, CA, USA), 0.15 μl of the forward and reverse 50 μM primers, 7.2 μl nuclease free water, and 5 μl of genomic DNA. PCR was performed on a MyCycler (Bio-Rad, Hercules, CA, USA) and included an initial denaturation step at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec, with a final elongation step at 72°C for 7 min. PCR products were analyzed on 2% agarose-gels, verified PCR products were purified using MinElute® PCR Purification Kit (Qiagen Inc, Valencia, CA, USA). Purified PCR products were measured spectrophotometrically, diluted with nuclease free water, mixed with primers then submitted to GeneWiz (GeneWiz Inc; South Plainfield, NJ, USA) for DNA sequencing and uploaded to GenBank (KC344734-KC344741).
Antimicrobial agents
Drugs used were isoniazid (INH), rifampin (RIF), ofloxacin (OFX; all from Sigma-Aldrich, St. Louis, MO, USA), amikacin (AMK; MP Biomedicals, Solon, OH, USA). INH, AMK were dissolved in sterile distilled water. RIF was dissolved in dimethyl
sulfoxide. OFX was dissolved in 0.1 N NaOH. All stock solutions were stored in single use aliquots at −80°C.

**Agar proportion method**

The drug susceptibility of H37Rv, XDR-TB and the population of H37Rv and XDR-TB in each mixture was tested in Middlebrook 7H10 agar (Difco) by standard procedure with slight modifications [14]. Briefly, 0.5 McFarland of purified H37Rv, XDR-TB and each H37Rv:XDR-TB mixture were diluted 10-fold serially in sterile distilled water and 10 μl of dilutions of 10\(^{-2}\) and 10\(^{-3}\) were spread onto M7H10 with and without drug and incubated at 37°C. Critical concentrations endorsed by CLSI were used [14]. Results were read 21 days after inoculation of media. At the critical concentration of each drug bacterial growth was counted to enumerate the % of resistant colonies, whereby >1% is defined as resistance.

**Results**

**Specificity of probes**

Primers and probes were utilized to detect wild-type and mutant sequence for each gene target based on the sequence of the XDR-TB isolate that was used (Table 1). This isolate was chosen because it harbored the Ser531Leu of *rpoB*, Ser315Thr of *katG*, A1401G of *rrs*, and Asp94Gly and Ser95Thr mutations of *gyrA* gene, the most common mutations reported for rifampin, isoniazid, injectables, and quinolones, respectively [13]. Specificity of each probe was stringently tested by singleplex PCR using high-concentration wild-type (H37Rv) and mutant (XDR-TB) DNA template (Fig. 1), amounting to approximately 28,600 cfu Tb cells/PCR reaction (Table S1). The *gyrA* wild-type probe showed high specificity for its target sequence, while *gyrA* mutant, *rpoB* mutant, both *katG* probes, and both *rrs* probes exhibited slight cross reactivity which was
manageable with threshold adjustment. The rpoB wild-type probe showed substantial cross reactivity to mutant target (Fig.1B).

Real-time PCR

The DNA of each H37Rv:XDR-TB mixture, as well as wild type (1:0) and mutant (0:1) controls, were amplified by real-time PCR for each gene individually with primers and both probes, using the thresholds of Figure 1. Real-time PCR detected mutant sequence at a 1:1 mixture of H37Rv:XDR-TB for all four genes (Fig. 2 shows katG as an example, for other genes see Figure S1). At a 10:1 mixture, mutants were weakly detected for all four genes. At 100:1 and 1000:1 mixtures, mutant sequence was not detected for any gene (Figure 2, Figure S1).

Sanger Sequencing

The DNA of each H37Rv:XDR-TB mixture was amplified with locus-specific primers and submitted for sequencing. Traces are shown in Figure 3. Mutant sequence was reported only at the 1:1 mixture. Subtle peaks of mutant nucleotide were visible at a 10:1 mixture.
Figure 4. Determination of dilution for digital PCR. The DNA of each H37Rv:XDR-TB mixture was diluted 10 fold serially and subjected to real-time PCR using the \textit{rpoB} primers and probes. The percent of PCR reactions that amplified at each dilution is shown. The target one-half genome equivalent DNA is the amount of DNA that yielded 50\% positive [10].

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Figure 5. Digital PCR. The DNA of each H37Rv:XDR-TB mixture was diluted 1:10000 and subjected to real-time PCR for each gene with both probes in 384 well plates. This figure shows representative 10:1 (\textit{katG}, \textit{gyrA}, \textit{rps}) or 100:1 (\textit{rpoB}) mixtures. Individual wells revealed detection with the wild-type probe (red traces), mutant probe (blue traces), both, or neither. qPCR thresholds were set per Figure 1 (red and blue horizontal lines for wild-type and mutant fluorophores, respectively). Cycling continued for 55 cycles (for \textit{katG}) due to dilute DNA template.

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### Table 2. Digital PCR.

| Gene | H37Rv: XDR-TB mixture | Number of Reactions | Number with no amplification | Number with wild-type sequence detected only | Number with both wild-type and mutant sequences detected | Number with mutant sequence detected only | % Mutant * |
|------|------------------------|---------------------|-----------------------------|---------------------------------------------|--------------------------------------------------------|------------------------------------------|------------|
| katG | 1:0                    | 384                 | 39                          | 345                                         | 0                                                      | 0                                        | 0          |
|      | 1:1                    | 384                 | 33                          | 81                                          | 262                                                    | 8                                        | 39.6       |
|      | 10:1                   | 384                 | 99                          | 243                                         | 25                                                     | 17                                       | 10.4       |
|      | 100:1                  | 384                 | 115                         | 260                                         | 6                                                      | 3                                        | 2.2        |
|      | 1000:1                 | 384                 | 156                         | 228                                         | 0                                                      | 0                                        | 0.0        |
|      | 0:1                    | 384                 | 42                          | 1                                           | 0                                                      | 341                                      | 99.7       |
| rpoB | 1:0                    | 384                 | 64                          | 320                                         | 0                                                      | 0                                        | 0          |
|      | 1:1                    | 384                 | 25                          | 75                                          | 284                                                    | 0                                        | 39.6       |
|      | 10:1                   | 384                 | 106                         | 222                                         | 56                                                     | 0                                        | 10.1       |
|      | 100:1                  | 384                 | 130                         | 250                                         | 2                                                      | 2                                        | 1.2        |
|      | 1000:1                 | 384                 | 202                         | 181                                         | 0                                                      | 1                                        | 0.5        |
|      | 0:1                    | 384                 | 35                          | 1                                           | 2                                                      | 346                                      | 99.4       |
| gyrA | 1:0                    | 384                 | 82                          | 302                                         | 0                                                      | 0                                        | 0          |
|      | 1:1                    | 384                 | 60                          | 82                                          | 136                                                    | 106                                      | 53.7       |
|      | 10:1                   | 384                 | 131                         | 217                                         | 16                                                     | 20                                       | 11.1       |
|      | 100:1                  | 384                 | 177                         | 206                                         | 1                                                      | 0                                        | 0.2        |
|      | 1000:1                 | 384                 | 162                         | 222                                         | 0                                                      | 0                                        | 0.0        |
|      | 0:1                    | 384                 | 69                          | 0                                           | 1                                                      | 314                                      | 99.8       |
| rrs  | 1:0                    | 384                 | 70                          | 314                                         | 0                                                      | 0                                        | 0          |
|      | 1:1                    | 384                 | 39                          | 59                                          | 195                                                    | 91                                       | 54.6       |
|      | 10:1                   | 384                 | 45                          | 270                                         | 65                                                     | 4                                        | 10.8       |
|      | 100:1                  | 384                 | 42                          | 334                                         | 8                                                      | 0                                        | 1.2        |
|      | 1000:1                 | 384                 | 67                          | 316                                         | 1                                                      | 0                                        | 0.2        |
|      | 0:1                    | 384                 | 53                          | 0                                           | 1                                                      | 330                                      | 99.8       |

*% mutant = [(n mixed population/2)+n mutant]/total n amplified.

**Since the *rpoB* wild-type probe showed substantial cross reactivity to mutant target (Fig.1B), detection of both wild-type and mutant *rpoB* probes in a single well was interpreted as presence of mutant sequence if fluorescence signal of mutant probe was higher than that of the wild-type probe, and interpreted as a mixed population if fluorescence signal of the mutant probe was equal or lower than that of the wild-type probe. Detection of wild-type only *rpoB* was interpreted as wild-type sequence.**

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The DNA of each H37Rv:XDR-TB mixture was then diluted 10 fold serially and subjected to real-time PCR using the rpoB primers and probes. The starting number of cells and input DNA are shown in Table S1. The percent of PCR reactions that amplified at each dilution was enumerated and shown in Figure 4. Specifically, at the 1/10000 dilutions approximately 40–60% of PCR reactions were positive for amplification, thus we chose this dilution for digital PCR as previously described [10]. To perform digital PCR the diluted DNA underwent PCR in 384 well plates for each gene individually with primers and both probes. This yielded wild-type, mutant, mixed, and negative results as indicated in Table 2 (see example traces on Figure 5). Positive wells were counted and the percentages of wild-type and mutant templates were calculated (% mutant = [(n mixed population/2) + n mutant]/total n amplified). All mixtures were simultaneously cultured by agar proportion method and percentage of resistant colonies enumerated for comparison. The % of mutant sequence detected by digital PCR mirrored the % of resistant colonies that were found by agar proportion in a much shorter timeframe (1 day versus 21), and yielded sensitivity down to as little as 0.1% mutant DNA in the mixture. This speed alone may yield clinical value, particularly as there are instances when a Tb isolate does not grow well during susceptibility testing.

### Discussion

This work demonstrates the principal of digital PCR for both detection and quantitation of heteroresistance in drug resistant tuberculosis. This method offered an extremely accurate representation of the percentage of resistant colonies that were found by agar proportion in a much shorter timeframe (1 day versus 21), and yielded sensitivity down to as little as 0.1% mutant DNA in the mixture. This speed alone may yield clinical value, particularly as there are instances when a Tb isolate does not grow well during susceptibility testing.

The work urges caution with interpretation of many PCR-based genotypic resistance tests, as is used in line probe, Xpert MTB/RIF, and sequencing methodologies. While these methods offer significant advantages in terms of speed and simplicity of interpretation, we found that qPCR-based probe methods (on DNA from an entire Tb isolate) could detect resistance only when present at a high amount (approximately 10% mixture). Sanger sequencing was only capable of detecting resistance at a 50/50 mixture (28–60% resistant colonies). Additionally, we examined a laboratory-developed rapid phenotypic DST assay that utilizes qPCR of mycobacteriophage [15] and that method detected resistance at the ~10% level as well (data not shown).

### Table 3. Comparison of digital PCR, agar proportion, real-time PCR, and PCR with Sanger sequencing.

| Gene | H37Rv: XDR-TB mixture | Agar proportion (% Resistant colonies) | Real-time PCR | PCR with Sequencing (Sanger) | Digital PCR (% Resistant colonies) |
|------|------------------------|---------------------------------------|---------------|------------------------------|-----------------------------------|
| katG | 1:1                    | R (60.2%)                             | R             | R                            | R (39.6%)                         |
|      | 10:1                   | R (13.8%)                             | R             | S                            | R (10.4%)                         |
|      | 100:1                  | R (3.9%)                              | S             | S                            | R (2.2%)                          |
|      | 1000:1                 | S (0.1%)                              | S             | S                            | S (0.0%)                          |
| rpoB | 1:1                    | R (29.4%)                             | R             | R                            | R (39.6%)                         |
|      | 10:1                   | R (6.9%)                              | R             | S                            | R (10.1%)                         |
|      | 100:1                  | R (2.8%)                              | S             | S                            | R (1.2%)                          |
|      | 1000:1                 | S (0.1%)                              | S             | S                            | S (0.5%)                          |
| gyrA | 1:1                    | R (28.3%)                             | R             | R                            | R (53.7%)                         |
|      | 10:1                   | R (2.2%)                              | R             | R                            | R (11.1%)                         |
|      | 100:1                  | S (0.5%)                              | S             | S                            | S (0.2%)                          |
|      | 1000:1                 | S (0.0%)                              | S             | S                            | S (0.0%)                          |
| rrs  | 1:1                    | R (62.1%)                             | R             | R                            | R (54.6%)                         |
|      | 10:1                   | R (7.9%)                              | R             | S                            | R (10.8%)                         |
|      | 100:1                  | R (2.7%)                              | S             | S                            | R (1.2%)                          |
|      | 1000:1                 | S (0.0%)                              | S             | S                            | S (0.2%)                          |

Correlation with agar proportion: 81% (13/16) 56% (9/16) 100% (16/16)

R = resistant. S = susceptible. Agar proportion resistance defined as >1% resistant colonies, and the actual percentage of resistant colonies is shown in parentheses. Digital PCR resistance defined as >1% of PCR reactions indicating mutant sequence, with the actual percentage of PCR reactions with mutant sequence shown in parentheses. Real-time PCR resistance defined as mixed populations of wild-type and mutant sequence. Sequencing defined resistance as evidence of mutant sequence by chromatogram.

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| H37Rv: XDR-TB mixture | Starting number of Tb cell in spiked sputum (cfu/ml) | Appropriate DNA dilution | Number of Reactions | Number with no amplification | Number with wild-type sequence detected only | Number with both wild-type and mutant sequences detected | Number with mutant sequence detected only | % Mutant |
|------------------------|-----------------------------------------------------|--------------------------|---------------------|----------------------------|---------------------------------------------|------------------------------------------------|---------------------------------------------|---------|
| 1:1                    | $1.88 \times 10^5$                                  | 1/1000                   | 384                 | 71                         | 40                                          | 93                                             | 180                                          | 72.4    |
| 10:1                   | $1.03 \times 10^5$                                  | 1/1000                   | 384                 | 235                        | 107                                         | 14                                             | 28                                           | 23.5    |
| 100:1                  | $9.5 \times 10^5$                                   | 1/1000                   | 384                 | 282                        | 99                                          | 0                                              | 3                                            | 2.9     |
| 1000:1                 | $9.4 \times 10^5$                                   | 1/1000                   | 384                 | 310                        | 73                                          | 1                                              | 0                                            | 0.7     |
| 1:1                    | $1.88 \times 10^3$                                  | 1/100                    | 384                 | 111                        | 38                                          | 53                                             | 182                                          | 76.4    |
| 10:1                   | $1.03 \times 10^3$                                  | 1/100                    | 384                 | 237                        | 110                                         | 8                                              | 29                                           | 22.4    |
| 100:1                  | $9.5 \times 10^3$                                   | 1/100                    | 384                 | 284                        | 98                                          | 0                                              | 2                                            | 2.0     |
| 1000:1                 | $9.4 \times 10^3$                                   | 1/100                    | 384                 | 295                        | 88                                          | 1                                              | 0                                            | 0.6     |
| 1:1                    | $1.88 \times 10^4$                                  | 1/10                     | 320                 | 112                        | 35                                          | 34                                             | 139                                          | 75.0    |
| 10:1                   | $1.03 \times 10^4$                                  | 1/10                     | 320                 | 213                        | 83                                          | 5                                              | 19                                           | 20.1    |
| 100:1                  | $9.5 \times 10^4$                                   | 1/10                     | 320                 | 228                        | 88                                          | 2                                              | 2                                            | 3.3     |
| 1000:1                 | $9.4 \times 10^4$                                   | 1/10                     | 320                 | 235                        | 84                                          | 1                                              | 0                                            | 0.6     |
| 1:1                    | $1.88 \times 10^3$                                  | undiluted                | 27                  | 3                          | 3                                           | 6                                              | 15                                           | 75.0    |
| 10:1                   | $1.03 \times 10^3$                                  | undiluted                | 27                  | 11                         | 12                                          | 0                                              | 4                                            | 25.0    |
| 100:1                  | $9.5 \times 10^3$                                   | undiluted                | 27                  | 21                         | 6                                           | 0                                              | 0                                            | 0.0     |
| 1000:1                 | $9.4 \times 10^3$                                   | undiluted                | 27                  | 23                         | 4                                           | 0                                              | 0                                            | 0.0     |

*Since the rpoB wild-type probe showed substantial cross reactivity to mutant target (Fig.1B), detection of both wild-type and mutant rpoB probes in a single well was interpreted as presence of mutant sequence if fluorescence signal of mutant probe higher than that of the wild-type probe, and interpreted as a mixed population if fluorescence signal of the mutant probe was equal or lower than that of wild-type probe. Detection of wild-type only rpoB was interpreted as wild-type sequence.

*the dilution that yielded approximately 50% PCR positive.

% mutant = [(n mixed population/2)+n mutant]/total n amplified.

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Our primary purpose of this work was to detect heteroresistance in isolates; however the digital PCR method also worked directly on sputum with sufficient Tb quantity (~10³ cfu Tb/ml sputum, Table 4). We also tested the system on nontuberculous mycobacteria and there was little positivity with most primers and probes across most NTM species (details in Table S2). This is not surprising, since the resistance determining region of \( \text{rpoB} \) is outside the variable region used for detecting other Mycobacterium species [16]. However given some cross-reactivity with other species one should use caution if using the assays on a mixed mycobacterial isolate or specimen.

The question, of course, is what is the clinical significance of low-level resistance and how common is the phenomena. Data on clinical significance is limited in this area and needs further study, particularly for second line drugs. However some studies in MDR-TB endemic settings have found heteroresistance to be common, on the order of 30% across several genes [6]. Therefore this is an area of great relevance given increases in MDR-TB globally, the proliferation of PCR-based genotypic assays in the clinical setting, and the potential risk of reporting false susceptibility with these methods should low-level resistance be present.

This was a study to prove the principal of digital PCR, and as such there were limitations. We used a single XDR strain with the most common \( \text{katG} \), \( \text{rpoB} \), \( \text{gyrA} \), \( \text{rrs} \), and \( \text{mrr} \) mutations, thus this assay would function on a Tb isolate with heteroresistance due to any of these mutations, but does not interrogate others. We chose our XDR strain because it harbored mutations at these key areas, but there are a diversity of other areas [13] that would need to be probed should one wish to devise a broadly applicable clinical diagnostic. Future work includes broadening the assay to incorporate more mutations and evaluating the performance of the assay on incoming clinical isolates against a range of conventional susceptibilities and against patient outcome. As commercial digital PCR platforms are becoming more available, we think this method offers great promise for quantification of drug resistant Tb.

Supporting Information

**Figure S1** Real-time PCR of undiluted DNA. The undiluted DNA of each H37Rv:XDR-TB mixture underwent real-time PCR for each gene with both probes. \( \text{rpoB} \), \( \text{gyrA} \), and \( \text{rrs} \) are shown, with detection of mutant sequence (blue trace) at various H37Rv:XDR-TB mixtures. qPCR thresholds were set per Figure 1 (red and blue horizontal lines for wild-type and mutant fluorophores, respectively).

**Table S1** Starting number and amount of cells in each PCR well for each diluted DNA template.

**Table S2** Specificity of each probe versus non-tuberculous mycobacteria (NTM).

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Author Contributions

Conceived and designed the experiments: SP SF EH. Performed the experiments: SP SS. Analyzed the data: SP EH. Contributed reagents/materials/analysis tools: SF. Wrote the paper: SP EH.

References

1. WHO (2011) Global tuberculosis control. WHO report 2011: World Health Organization, Geneva, Switzerland.
2. Rinder H (2003) Hetero-resistance: an under-recognised confounder in diagnosis and therapy? J Med Microbiol 50: 1018–1020.
3. Morand B, Muhlemann K (2002) Heteroresistance to penicillin in Streptococcus pneumoniae. Proc Natl Acad Sci U S A 104: 14098–14103.
4. Rinder H, Mieskes KT, Loscher T (2001) Hetroresistence in Mycobacterium tuberculosis. Inf J Tuberc Lung Dis 5: 339–345.
5. Chakravorty S, Aldeghamni B, Thomas K, Lee JS, Lee EG, et al. (2011) Rapid detection of fluoroquinolone-resistant and heteroresistant Mycobacterium tuberculosis by use of sloppy molecular beacons and dual melting-temperature codes in a real-time PCR assay. J Clin Microbiol 49: 932–940.
6. Tolani MP, D’Ouza DTB, Mistry NF (2012) Drug resistance mutations and heteroresistance detected using the GenoType MTBDRplus assay and their implication for treatment outcomes in patients from Mumbai, India. BMC Infectious Diseases 12.
7. Zhang X, Zhao B, Liu L, Zhu Y, Zhao Y, et al. (2012) Subpopulation analysis of heteroresistance to fluoroquinolone in Mycobacterium tuberculosis isolates from Beijing, China. J Clin Microbiol 50: 1471–1474.
8. Hofmann-Thiel S, van Ingen J, Feldmann K, Turaev L, Uzakova GT, et al. (2009) Mechanisms of heteroresistance to isoniazid and rifampin using fluorogenic probes. J Antimicrob Chemother 64: 1300–1304.
9. Blakemore R, Story E, Helb D, Kop J, Banada P, et al. (2010) Evaluation of the analytical performance of the Xpert MTB/RIF assay. J Clin Microbiol 48: 2495–2501.
10. Vogelstein B, Kinzler KW (1999) Digital PCR. Proc Natl Acad Sci U S A 96: 9236–9241.
11. Espasa M, Gonzalez-Martinez J, Alcaide F, Aragon LM, Lonca J, et al. (2005) Direct detection in clinical samples of multiple gene mutations causing resistance of Mycobacterium tuberculosis to isoniazid and rifampicin using fluorogenic probes. J Antimicrob Chemother 55: 860–865.
12. Yesilkaya H, Meacci F, Niemann S, Hilleman D, Rusch-Gerdes S, et al. (2006) Evaluation of molecular-Beacon, TaqMan, and fluorescence resonance energy transfer probes for detection of antibiotic resistance-conferring single nucleotide polymorphisms in mixed Mycobacterium tuberculosis DNA extracts. J Clin Microbiol 44: 3826–3829.
13. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metlock B, et al. (2011) Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob Agents Chemother 55: 2032–2041.
14. CLSI (2011) Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard-Second Edition. CLSI document M24-A2. Wayne, PA: Clinical and Laboratory Standards Institute.
15. Pholwat S, Elshaaie B, Founglaakda S, Kelly B, Houpt E (2012) Real-time PCR using mycobacteriophage DNA for rapid phenotypic drug susceptibility results for Mycobacterium tuberculosis. J Clin Microbiol 50: 754–761.
16. Lee H, Bang HE, Bai GH, Cho SN (2003) Novel polymorphic region of the rpoB gene containing Mycobacterium species-specific sequences and its use in identification of mycobacteria. J Clin Microbiol 41: 2213–2218.