Infection and Drug Resistance

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ORIGINAL RESEARCH

Highly Sensitive Detection of Isoniazid Heteroresistance in *Mycobacterium Tuberculosis* by Droplet Digital PCR

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Purpose: The drug resistance of *Mycobacterium tuberculosis* constitutes a major public health threat. Existing approaches make it challenging to detect low levels of drug-resistant TB, also known as heteroresistance (HR), in a population. The recently found droplet digital PCR (ddPCR) is a sensitive method for determining the precise amount of nucleic acid in a sample. We used ddPCR to test the *Mycobacterium tuberculosis* heteroresistance because it delivers more exact quantitative data without the need for a reference curve.

Patients and Methods: A TaqMan-MGB probe mutation detection assay was developed in order to determine the mutant and wild-type sequences of the isoniazid resistance *katG* (315) gene. We produced heteroresistant MTB combinations, which were subsequently identified by ddPCR, qPCR, and MeltPro/INH. In addition, 21 clinical sputum samples with positive smears were used to validate each method's capacity to determine HR in sputum.

Results: We discovered that ddPCR can detect mutant sequences in as few as 0.01% of a combination. DeepMelt TB/INH, which is less sensitive in comparison, cannot detect HR with high resolution and requires a mutation rate of 50% to identify. qPCR likewise has a high resolution of 0.02%, but unlike ddPCR, it cannot determine the exact number of mutations. Our assay is applicable to sputum as well. ddPCR found a *katG* 315 substitution in two sputums with extremely low values of HR (0.26% and 0.14%). In 21 samples of clinical sputum, the HR prevalence of INH was 9.5%.

Conclusion: This work demonstrates that a well-designed ddPCR HR detection test can detect low levels of HR with high accuracy and consistency and gives new information for the clinical diagnosis of drug resistance.

Keywords: *Mycobacterium tuberculosis*, drug-resistant, isoniazid, heteroresistance

Introduction

Drug-resistant tuberculosis (TB) continues to represent a threat to public health. Multidrug-resistant tuberculosis (MDR-TB) is of most concern as it is defined by resistance to the two most effective first-line drugs, isoniazid (INH) and rifampin (RIF). The treatment of MDR-TB and rifampicin-resistant tuberculosis (RR-TB) must be escalated to second-line drugs. In addition, INH-resistant TB without concurrent RIF resistance accounts for 7.2% of new TB cases, which is higher than the estimated percentage of RR-TB cases.[1] Several studies have considered INH mono-resistance as a precursor to MDR-TB, and it has been observed before RIF resistance.[2–5]

Heteroresistance (HR) in *Mycobacterium tuberculosis* (MTB) is characterized as the presence of heterogeneous populations of drug-susceptible and -resistant bacteria in a single clinical specimen obtained from TB patients.[6] HR may emerge from a mixed infection, in which resistant and susceptible strains infect the same individual, or from a single clone changing from a susceptible to a resistant strain as a result of genetic mutation under antibiotic stress.[7] HR in MTB to several drugs have been reported, and it is believed that HR contributes to worsening tuberculosis outcomes.[8–10] HR is a crucial phase in the evolution of drug resistance in bacterial isolates. Current molecular approaches make it challenging to discover HR. Xpert MTB/RIF,[11] the global standard genetic diagnostic tool for

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drug-resistant *MTB*, can only detect resistance when the combination contains at least 65.6% mutant DNA. It has been discovered that culture-based phenotypic drug susceptibility testing (DST) is the most sensitive method, finding 1% of clinically significant resistant bacteria. In contrast, procedures such as line probe assays and Sanger sequencing can not achieve this result.[12] Deep sequencing[13,14] and mass array-based iPLEX gold assays[15] can detect 1% HR, but they require costly equipment and sophisticated procedures, limiting their practical application. In a recent study of multiple melting curves, the HR detectability was found to be between 20 and 30% the same as that of Sanger sequencing.[16]

The droplet digital polymerase chain reaction (ddPCR) represents the third generation of polymerase chain reaction (PCR). The ability of ddPCR to accurately detect and quantify low abundant targets has resulted in a rapid expansion of its applications in the detection of various pathogens.[17,18] ddPCR uses microfluidics to separate a standard PCR reaction into a water/oil emulsion of thousands of nanodroplets, essentially allowing one to perform thousands of independent PCR reactions in a single tube. Following PCR in a standard thermocycler, the number of positive droplets is determined using flow cytometry, and the number of copies of DNA template in the initial reaction can be inferred using Poisson statistics. The majority of INH-resistant MTB isolates are thought to carry *katG* S315T mutations, with a rate as high as 89%.[19] In this study, we selected S315T as the detection locus, developed a ddPCR mutation detection assay, and evaluated the capacity of ddPCR to detect and quantify HR in MTB mixed populations and sputum.

Material and Methods

Mycobacterial Strains

This study employed MTB H37Rv and a clinical INH-resistant MTB strain (100% resistant to isoniazid as determined by the agar proportion method), with the most common INH resistance-related mutation of katG315 (AGC-ACC). Strains obtained from the National Tuberculosis Reference Laboratory (NTRL).

Agar Proportion Method

The 7H10 agar proportion method was used to test the susceptibility of MTB to anti-tuberculosis drugs. The INH critical concentration was 1.0μg/mL. The procedure cites the Association for Clinical and Laboratory Standards' recommendation.

DNA Extraction

The cetyltrimethylammonium bromide (CTAB) sodium chloride method was used to extract genomic DNA (gDNA), and the concentration of the DNA was measured with a Qubit™ 2 Fluorometer (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's guidelines.

Reference Panel

The nucleic acids of H37Rv (WT) and INH-resistant (MT) were mixed as 1:1 and subjected to a 5-fold gradient dilution in order to identify the upper limit concentration of DNA template in the ddPCR reaction system and validate the specificity of the mutation detection assays. The initial total concentration of the mixed DNA was 550ng/mL. Additionally, standard curves for qPCR and ddPCR were generated by using a set of reference panels.

HR

By mixing WT and MT nucleic acids, artificial HR test panels are created. A series of MT DNA dilutions were made in the same WT DNA background. The percentages of the MT were 100.00%,50.00%,33.33%,20.00%,11.11%,5.88%,3.03%,1.54%,0.78%, 0.39%,0.19%,0.10%,0.05%,0.02%,0.01%, 0.00% respectively.

Oligonucleotides

Both the wild allele (in HEX) and the mutant allele (in FAM) may be identified using a single set of primers and two competing probes. Primer Express 3.0.1 was used to design *katG* S315T primers and probes. Table 1 shows each sequence.

Table 1 Primer and Probe Sequences

Primers and Probes	Sequences
katG-F	5'-TGGAAGAGCTCGTATGGCAC-3'
katG-R	5'-AGGAAACTGTTGTCCCATTTCG-3'
katG-MT	5'-FAM-CGATCACCACCGGC-MGB-3'
katG-WT	5'-HEX-CGATCACCAGCGGC-MGB-3

Digital Droplet PCR

The ddPCR mix (20μℓ) consisted of 10μℓ of ddPCR supermix for probes (2X) (Biorad, Carlsbad, CA, USA), 2μℓof 10 mM of each forward and reverse primer, 0.3μℓ of 10 mM of MT probe(FAM) and 0.7μℓ of 10 mM of WT probe(HEX), 5μℓ nuclease-free water and DNA template. All components were defrosted at room temperature, properly mixed by tilting the tube several times to achieve homogeneity, then quickly centrifuged. An initial reaction pool slightly larger than 20μℓ (22μℓ) was created to ensure that 20 μL of the mixture was transferred to the DG8 cartridge. Annealing temperatures optimized for ddPCR assays were performed on a CFX96 (Biorad CFX, Carlsbad, CA, USA), confirming the amplification procedure as initial denaturation at 95°C for 10 min, followed by 42 cycles denaturation at 95°C for 15 sec, and annealing and extension at 58°C for 30 sec. Ramp rate of 2 °C/sec was used to ensure that each droplet reaches the correct temperature at every step of the cycle. Using a QX200 Droplet Reader, the endpoint fluorescence signal of each droplet in the emulsion was measured immediately after processing. In duplicate, assays were conducted for each sample and target.

qPCR

PCR mixtures (18μℓ) consisted of 10μℓ of TaqMan™ Fast Advanced Master Mix (2X) (Thermo Fisher, Valencia, CA, USA), 1.5μℓ of 10 mM of each forward and reverse primer, 0.3μℓ of 10 mM of MT probe (FAM) and 0.7μℓ of 10 mM of WT probe (HEX), 4μℓ nuclease-free water, and DNA template. qPCR was performed on a CFX 96 (Biorad CFX, Carlsbad, CA, USA) with initial denaturation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec, and annealing and extension at 60°C for 30 sec.

MeltPro TB/INH

The DeepMelt TB/INH assay (ZEESAN, China) was utilized to detect all commonly observed mutations linked with INH resistance, such as the *katG* 315 mutation.[20] The reaction mixture contains 5μℓof template and 20μℓof PCR mixture. On a SLAN-96S real-time PCR system, amplification and melting curve analyses were performed.[20]

Sputum Samples

To confirm the capacity to measure and identify HR in sputum samples, 21 smear-positive clinical sputum samples from Anhui Provincial Chest Hospital were obtained (Hefei, Anhui, China). Using the Sputum DNA Isolation Kit (ZEESAN, China), DNA was extracted according to the manufacturer's instructions and sent to the NTRL.

Data Analyses

Fluorescence intensity signals for each droplet were saved as.csv files from the QuantaSoft Droplet Reader software (version 1.7.3, BioRad). Using ddPCR mutation detection assays on a mixed sample, the number of droplets in each cluster is used to calculate the concentrations of the WT and MT templates. The QuantSoft Droplet Reader software automatically calculates the threshold at which a droplet is deemed positive, assuming the data meets specified quality metrics. The threshold value can also be manually adjusted per well or plate referring to the threshold value, which is determined based on the results of three control wells (NTC, WT-only positive, and 1% MT positive) that can clearly separate the negative, single positive, and double positive droplets (Figure S1). GraphPad Prism 8 software was used to generate the graph.

Ethics Approval

The Ethics Committee of the Chinese Center for Disease Control and Prevention gave their blessing to proceed with this investigation. This study used drug-resistant surveillance strains that were collected in the province of Anhui between the years 2021 and 2022. Before any sputum samples were taken, each patient was required to sign a document indicating that they had read and understood the procedure.

Results

Assay Specificity and Optimum DNA Template Concentration for the ddPCR

What is critical is the capacity to distinguish mutant sequences from wild-type sequences. By adjusting the annealing temperature of the PCR and modifying the concentration of the template, all the droplets were dispersed as uniformly as possible throughout the four bins.

To detect mutations, we tailored our studies with a dual probe that could detect up to 0.45ng of MTB gDNA (80,000 copies) in a 20$\mu\ell$ reaction (Figure 1A).

A No Call will be returned with an inadequate number of empty droplets and too many positive droplets to apply Poisson statistics. Higher template concentrations (Figure 1A) made it more difficult to discriminate between double-positive and wild-type clusters than lower template concentrations (Figure 1B and C).

Standard Curve of qPCR and ddPCR

To determine the starting copy number of gDNA, the formula MTB-specific gene copy number = (concentrate in ng$\times 6.022\times 10^{23}$)/ (genome length$\times 10^{9}\times 650$) was used. For qPCR, the absolute starting copy numbers (converted to $\log_{10}$) was used to make linear correlation with the Ct value. For ddPCR, both of measured concentrations and expected concentrations were converted to the $\log_2$ value and used to perform the linear correlation analysis.

For qPCR, a linear association was detected between the starting copy number and the Ct value (linear dynamic range of 5.5–433,256.9 copies/total reaction volume tested) (R^2 = 0.9858 and R^2 = 0.9948) (Figure 2A). Strong linear correlations were obtained for WT and MT ddPCR reactions based on the DNA template range of 1.3–103,649.9 copies per reaction volume, with R2 = 0.9912 and R2 = 0.9943, respectively (Figure 2B). Even at a gDNA concentration of 0.066 copies/$\mu\ell$, the standard curve for ddPCR was normal. Therefore, the ddPCR had a detection sensitivity of 1.45 copies in a prepared 22$\mu\ell$reaction volume, whereas qPCR had a detection sensitivity of 5.5 copies (Figure 2).

Analytical Performance of ddPCR, qPCR and MeltPro-TB/INH

Figure 3 shows the resolution of small copy number changes using ddPCR for a series of double dilutions of MT ranging from 2274 copies/$\mu\ell$ to 0.21 copies/$\mu\ell$, with a background average of 2225 copies/$\mu\ell$ for WT (mutation rate ranging from 50% to 0.01%).

The limit of detection for HR was 0.01% for ddPCR, 0.02% for qPCR, and 33.33% for DeepMelt TB/INH. The mutation rate determined by ddPCR demonstrated an excellent correlation with the predicted mutation rate of the mixture (Table 2). In the qPCR experiment where the HR was 0.02%, the repeat detection CT values were 34.73, 35.19, and 33.98, respectively. qPCR and DeepMelt TB/INH only provided binary results (“Resistant” or “Susceptible”).

Performance of Isoniazid Resistance Detection for Different Assays on Sputum

Six sputum samples with katG 315 substitution were detected by ddPCR with varying HR levels ranging from 0.14% to 100%. MeltPro/INH detected only 4/6 sputum samples with 100% katG 315 substitution mutation rate detected by ddPCR. Two sputums with very low levels of HR (0.26% and 0.14%) detected by ddPCR were missed by MeltPro/INH. qPCR detected 4/6 sputum samples with katG 315 substitution, of which 3 samples had a 100% mutation rate and one sample had a 0.14% mutation rate. One sputum (ID 41902) with a 0.26% mutation rate and one sample (ID 41901) with a 100% mutation rate but a very low concentration of MT DNA in the sputum (2.37 copies/ul) was missed by qPCR (Table 3).

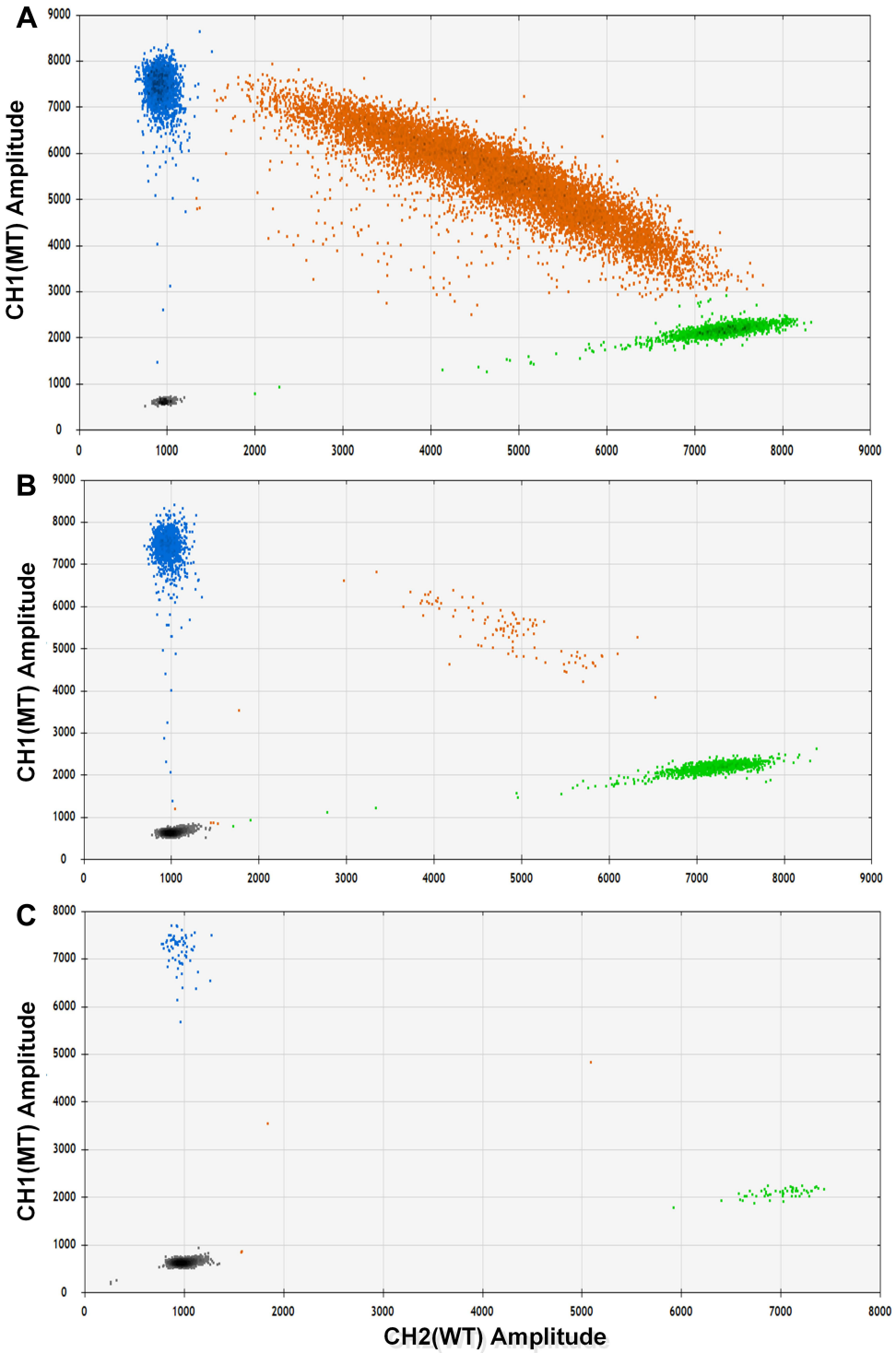

Figure 1 The effect of DNA template concentration on ddPCR detection specificity. Four clusters of droplets in 2-D plots: (1) Double-negative droplets without targeted DNA templates (gray); (2) WT-only positive droplets (green); (3) MT-only positive droplets (blue); and (4) Double-positive droplets with both WT and MT DNA templates (Orange). (**A**), Mixed DNA 0.55 ng (WT/MT 1:1); (**B**) Mixed DNA 0.022 ng (WT/MT 1:1); and (**C**), Mixed DNA 0.00018 ng (WT/MT 1:1).

Discussion

The transition from susceptible to monoresistant and/or MDR begins with HR.[21] All types of resistance are to blame when a treatment does not work, so it's important to know where HR comes from, how it spreads, and how to find it. Our study initially explored the ability of ddPCR to identify and quantify HR in drug-resistant TB. This method offered

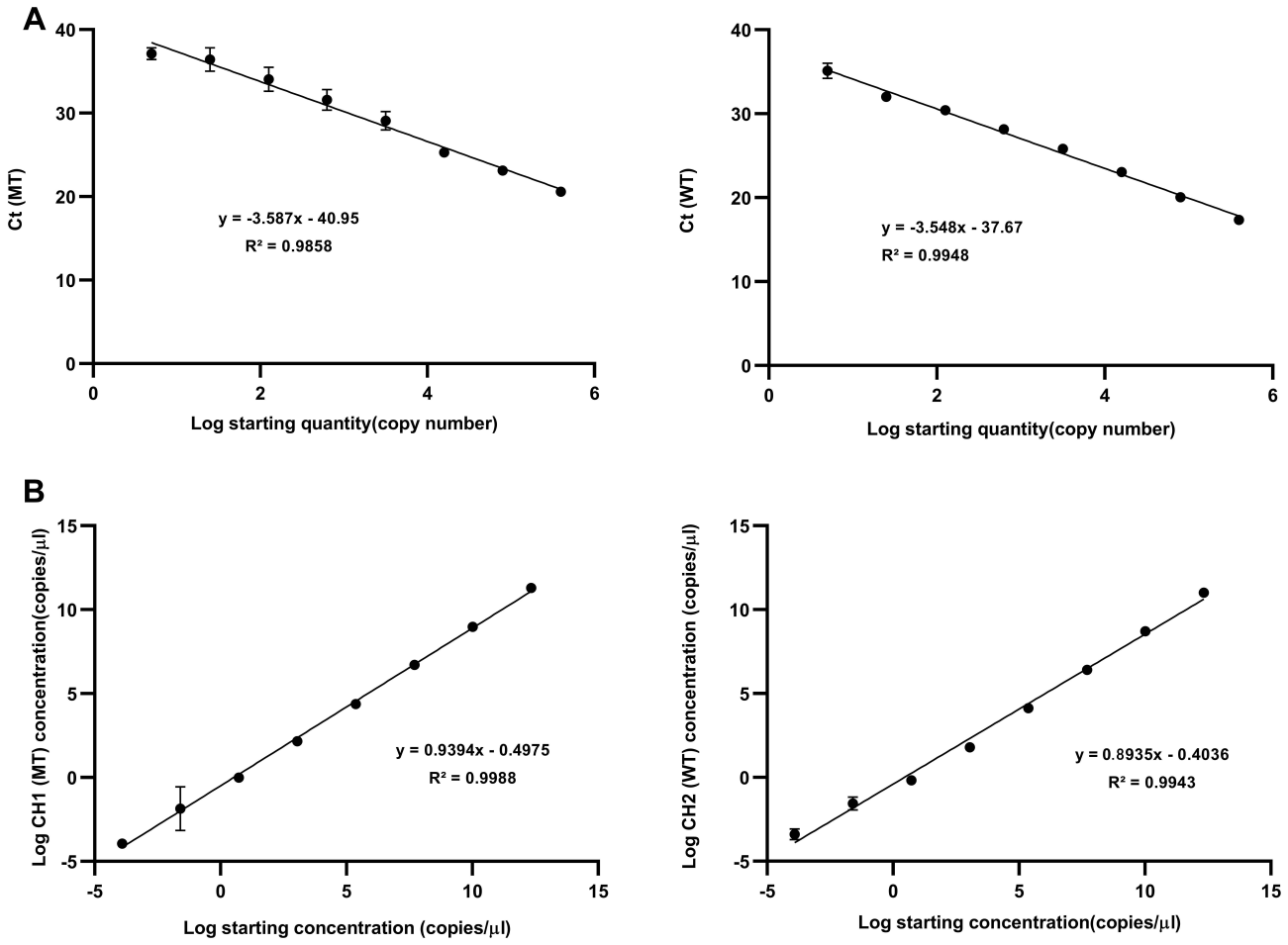

Figure 2 Liner correlation between expected starting DNA and measured DNA using qPCR and ddPCR. (**A**). Starting quantity (converted to log_{10}) were plotted on the X-axis versus measured Ct values of qPCR on the Y-axis for MT (Left) and WT (Right); (**B**) Starting concentration (converted to log_2) were plotted on the X-axis versus measured values of ddPCR (converted to log_2) on the Y-axis for MT(Left) and WT (Right).

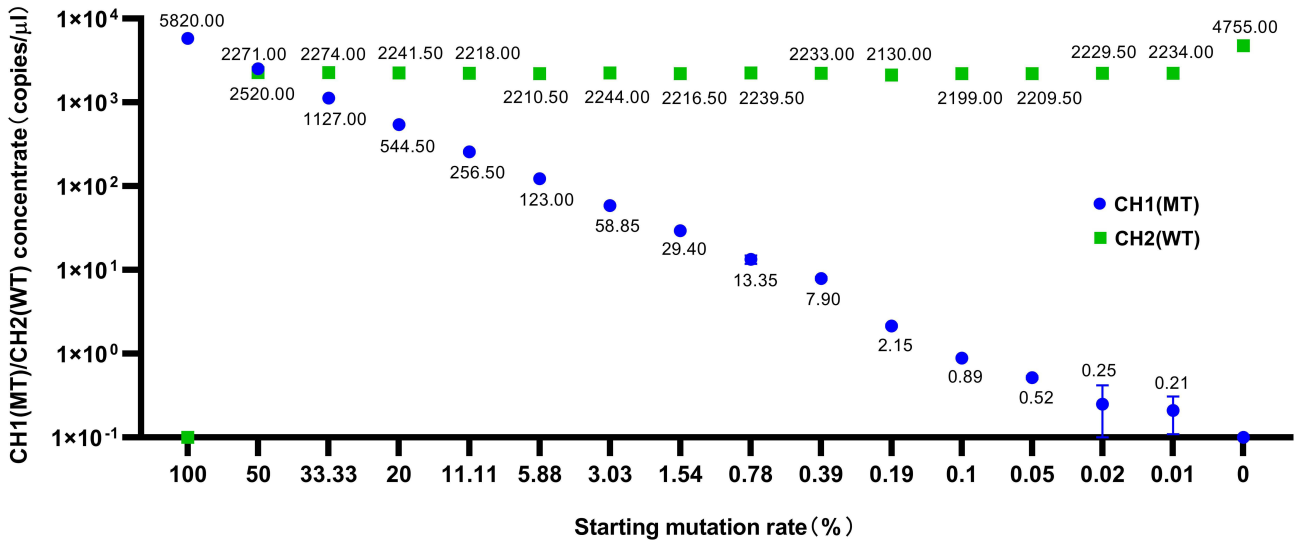

Figure 3 MT and WT concentrations measured by ddPCR at various hetro-resistance level. Mean value of two repeated tests. It includes Standard Deviation error bars.

Table 2 Comparison of ddPCR, qPCR, and MELTPro-TB/INH

Starting Mutation Rate MT/(WT+MT)%	qPCR	MELTPro-TB/INH	ddPCR		
			MT Copies/Well (Mean)±SD	WT Copies/Well (Mean)±SD	Mutation Rate MT/(WT+MT)%
100.00%	R	R	116400.00±5374.01	0.00±0.00	100%
50.00%	R	R	50400.00±848.53	45,420.00±254.56	52.60%
33.33%	R	R	22540.00± 282.84	45,480.00±84.85	33.14%
20.00%	R	S	10890.00±212.13	44,830.00±551.54	19.54%
11.11%	R	S	5130.00±183.85	44,360.00±339.41	10.37%
5.88%	R	S	2460.00±0.00	44,210.00±127.28	5.27%
3.03%	R	S	1177.00±18.38	44,880.00±678.82	2.56%
1.54%	R	S	588.00±2.83	44,330.00±890.95	1.31%
0.78%	R	S	267.00±29.70	44,790.00±777.82	0.59%
0.39%	R	S	158.00±5.66	44,660.00±424.26	0.35%
0.19%	R	S	43.00±1.41	42,600.00±113.14	0.10%
0.10%	R	S	17.80±0.28	43,980.00±395.98	0.04%
0.05%	R	S	10.30±0.42	44,190.00±353.55	0.02%
0.02%	R	S	5.00±3.93	44,590.00±14.14	0.01%
0.01%	S	S	4.20±1.96	44,680.00±707.11	0.01%
0.00%	S	S	0.00±0.00	95,100.00±4102.00	0.00%

Note: qPCR resistance defined as 15<Ct<35 indicating mutant sequence.
Abbreviations: R, resistant; S, susceptible.

Table 3 Detection of Isoniazid Resistance in Sputum Using Melt/INH Pro and ddPCR

ID	MeltPro/INH		qPCR	ddPCR				
			katG S315T	MT(copies/$\mu\ell$)		WT(Copies/$\mu\ell$)		HR LEVEL *katG* S315T
	R/S	*Loci*	R/S	PCR MIX(Mean)	Sputum* (Mean) ±SD	PCR MIX(Mean)	Sputum* (Mean) ±SD	MT/(WT+MT) %
41,901	R	*katG* 315	Not detected	0.33	3.63±0.62	0.00	0.00±0.00	100.00%
41,902	S	-	S	0.22	2.37±0.08	82.75	910.25±38.11	0.26%
41,904	R	*ahpC* promoter	R	0.44	4.79±0.70	305.50	3360.50±272.24	0.14%
42,101	S	-	S	0.00	0.00±0.00	12.70	139.70±.7.78	0.00%
42,103	R	*inhA* promoter	S	0.00	0.00±0.00	132.50	1457.50±23.33	0.00%
42,104	R	*inhA* promoter; *katG* 315	R	43.90	482.90±32.67	0.00	0.00±0.00	100.00%
42,105	R	*katG* 315	R	17.45	191.95±13.22	0.00	0.00±0.00	100.00%

(*Continued*)

Table 3 (Continued).

ID	MeltPro/INH		qPCR	ddPCR				
			katG S315T	MT(copies/$\mu\ell$)		WT(Copies/$\mu\ell$)		HR LEVEL *katG* S315T
	R/S	*Loci*	R/S	PCR MIX(Mean)	Sputum* (Mean) ±SD	PCR MIX(Mean)	Sputum* (Mean) ±SD	MT/ (WT+MT) %
42,106	S	-	Not detected	0.00	0.00	0.21	2.31±0.16	0.00%
42,108	S	-	S	0.00	0.00±0.00	14.05	154.55±2.33	0.00%
42,109	S	-	S	0.00	0.00±0.00	29.55	325.05±22.56	0.00%
42,110	S	-	S	0.00	0.00±0.00	3.60	39.60±3.11	0.00%
42,111	R	*ahpC* promoter; *katG* 315	R	24.35	267.85±17.89	0.00	0.00±0.00	100.00%
42,112	S	-	S	0.00	0.00±0.00	1.20	13.20±3.11	0.00%
42,113	R	*ahpC* promoter;*inhA* promoter	S	0.00	0.00±0.00	12.40	136.40±3.11	0.00%
42,114	R	*inhA* promoter	S	0.00	0.00±0.00	1.90	20.90±0.00	0.00%
42,115	R	*inhA* promoter	S	0.00	0.00±0.00	215.50	2370.50±54.45	0.00%
42,116	S	-	S	0.00	0.00±0.00	25.70	282.70±17.11	0.00%
42,117	S	-	S	0.00	0.00±0.00	0.83	9.13±2.64	0.00%
42,118	R	*ahpC* promoter;*inhA* promoter	S	0.00	0.00±0.00	31.75	349.25±27.22	0.00%
42,119	S	-	S	0.00	0.00±0.00	65.65	722.15±0.78	0.00%
42,120	S	-	S	0.00	0.00±0.00	22.00	242.00±37.34	0.00%

Note: *Concentration (copies/$\mu\ell$) in sputum = (PCR MIX concentration*reaction volume)/DNA template volume.
Abbreviations: R, resistant; S, susceptible.

excellent accuracy, with a sensitivity as low as 0.01% mutant DNA in a mixture. In order to conduct duplex tests, two targets were amplified concurrently in a single reaction. This reduced technical errors, such as cumulative pipetting inaccuracy, and allowed the determination of smaller differences compared to parallel uniplex reactions.[22] A recent work demonstrated the role of dPCR in the identification and quantification of HR in a mixed TB population, where dPCR was able to detect 0.1% of mutant sequences.[23]

For the qPCR-based probe method, we also observed a robust HR detection level of 0.02%. While the nature of qPCR makes quantification conceivable, our results indicate that accuracy and repeatability must be evaluated and improved before this approach can be utilized to determine the clinical feasibility of molecular quantification of MTB during the care of TB patients. The DeepMelt TB/INH assay is a closed-tube, dual-color, melting curve analysis-based, real-time PCR test that has been given approval by the Chinese Food and Drug Administration. It was made to find INH resistance mutations with a minimum HR level slightly below the parameters (>40%) included with the kit.[24]

The prevalence of HR varies from study to study. The prevalence of HR varies a lot, ranging from less than 1% to even higher than 20%.[7,16,19,25–30] Variation in sample size and detection techniques may be the most important variables contributing to variances in HR prevalence. Our research reveals the efficacy of ddPCR on sputum samples. In 21 clinical sputum samples, the prevalence of HR for INH *katG* 315 was 9.5% by ddPCR. The reason that MeltPro TB/INH missed the *katG* 315 mutation in two samples validated by ddPCR may be the relatively low proportion of isoniazid-resistant

strains in bacterial population, which was out of Melt/INH Pro's detection limit of 33.33% as shown in the artificial mixed DNA in the present study. The number of INH-resistant samples with mutations in the *katG* locus was almost equivalent to that of samples with mutations in the *inhA* promoter area. The most common mutations in the *katG* and *inhA* loci were identified by Campbell et al[31] and Rodwell et al[32] from the United States at codon 315 and C(−15)T, respectively. Also, the low quantitative copy number found by ddPCR in clinical sputum samples may be related to the way nucleic acids were extracted and how they were shipped. The sputum DNA was transported to the NTRL laboratory without a cold chain and lasted around 2 days. So the delay and temperature may affect the quality and quantity of DNA.

The following constraints apply to this research: We utilized a single INH-resistant strain with the most prevalent *katG* 315 mutation; hence, this test would only be applicable to a TB isolate with HR due to a *katG* mutation and not to other strains. Also, since only 11 strains were tested for clinical resistance to *katG* mutations, sensitivity estimates are not as accurate as they could be. This means that more validation trials are needed. Thirdly, no correlation analysis was conducted between TB clinical isolates and patient outcome.

In future studies, other mutations will be added, and the assay will be compared to a number of known susceptibilities and patient outcomes to see how well it works on clinical isolates.

Conclusion

ddPCR is an endpoint measurement that allows the quantification of nucleic acids without the use of standard curves and regardless of reaction efficiency. Our study demonstrates that well-designed ddPCR HR detection assays may detect low HR levels with high accuracy and good consistency, which can provide additional information for clinical diagnosis of drug resistance.

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Disclosure

The authors state that there are no competing interests related to this study.

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