Am. J. Trop. Med. Hyg., 95(6), 2016, pp. 1257–1264
doi:10.4269/ajtmh.16-0120

Rapid Detection of *Mycobacterium tuberculosis* Strains Resistant to Isoniazid and/or Rifampicin: Standardization of Multiplex Polymerase Chain Reaction Analysis

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Abstract. Drug susceptibility testing using molecular techniques can enhance the identification of drug-resistant *Mycobacterium tuberculosis*. Two multiplex real-time polymerase chain reaction (qPCR) assays were developed to detect the most common resistance-associated mutations to isoniazid (*katG*S315T, *inhA*-15C→T), and rifampicin (*rpoB*H526Y and *rpoB*S531L). To assess the species specificity of the qPCR, we selected 31 nontuberculous mycobacteria (NTM) reference strains belonging to 17 species from the public collection of mycobacterial cultures (BCCM/ITM). Additionally, we tested 17 isoniazid and/or rifampicin-resistant strains with other mutations in the target genes to assess mutation specificity. The limit of detection for all the targeted mutations was 20 bacilli/reaction. Multiplex 1 showed 90%, 95%, and 100% efficiency for wild type (WT), Mut *katGS*315T, and Mut *rpoBS*531L, respectively; whereas Multiplex 2 showed 97%, 94%, and 90% efficiency for WT, Mut *inhA*-15, and Mut *rpoBH*526Y, respectively. Three of 17 strains that presented other mutations in the target genes were identified as rifampicin resistant and only 3/31 NTM showed a similar melting temperature to *rpoB*L531 and/or *katG*T315 mutants. Thus, our proposed cascade of specific tuberculosis detection followed by drug resistance testing showed sensitivities for *katGS*315T, *rpoBS*531L, *rpoBH*526Y, and *inhA*-15 detection of 100%, 100%, 100%, and 96%, respectively; and specificities of 98%, 95%, 100%, and 100, respectively.

INTRODUCTION

Despite not being among the 10 leading causes of death, tuberculosis (TB) remains a global public health concern.[1] In 2013, 9.0 million people developed TB and 1.5 million died of the disease (including human immunodeficiency virus positive). TB mortality rate and prevalence has fallen worldwide by an estimated 41% and 36% respectively, between 1990 and 2014; however, the proportion of multidrug-resistant TB (MDR-TB) has remained almost the same. Globally, 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB in 2013, which means that around 480, 000 people developed MDR-TB.[2] Despite this significant burden, only a limited number of tests have been developed and implemented for the rapid diagnosis of TB. Further, since the majority of TB disease burden occurs in underdeveloped and resource-limited settings, the need for a cost-efficient method is paramount.

A significant obstacle in controlling TB is the amount of time required to reach a bacteriologically confirmed diagnosis. Due to the slow growth rate of *Mycobacterium tuberculosis*, the initial culture can take up to 6 weeks, with up to an additional 12 weeks to obtain drug susceptibility profiles for clinical isolates, depending on the techniques available in the laboratory. These labor-intensive methods can cause significant delays in identifying MDR cases, subsequent adjustment of treatment regimens, and initiation of epidemiological investigations.

The use of molecular techniques as drug susceptibility testing (DST) tools can enhance the identification of drug-resistant *M. tuberculosis*. Resistance to isoniazid (H) is a complex process. Mutations in *katG* (catalase-peroxidase), *inhA* (enoyl-ACP reductase), *kasA* (β-ketoacyl-ACP synthase), and *ndh* (NADH dehydrogenase) have been associated with isoniazid resistance, with mutations in *katG* (60–70%) and *inhA* or its promoter region prevailing (~10%).[3–6] Rifampicin (R), resistance is mostly (96%) due to mutations in an 81–(base pair) bp "hot-spot" region of the *rpoB* gene that encodes the β-subunit of RNA polymerase, especially in codons 531 (43–56%) and 526 (8–31%).[7–10]

Several molecular methods are available for detection of drug resistance mutations, including denaturing gradient gel electrophoresis, conformation-sensitive gel electrophoresis, temperature gradient capillary electrophoresis, denaturing high-performance liquid chromatography, high-density oligonucleotide arrays, and high-resolution melting analysis.[11–17] These methods vary in sensitivity and are either labor intensive, require sophisticated equipment to perform analyses, or present ambiguity in interpretation. Polymerase chain reaction (PCR)–based DNA sequencing of drug resistance–related genes is the most specific method to identify mutations.[18] However, due to the high cost of sequencing and the expertise and infrastructure required, it is not widely available, especially in resource-constrained settings, often high TB and MDR-TB burden areas with large numbers of samples requiring testing.

Since 2010, the disposable cartridge-based GeneXpert MTB/RIF (Cepheid, CA) commercial assay has been endorsed for TB diagnosis with subsidized pricing available to selected health-care providers in selected countries. However, in many resource-constrained settings, this subsidized pricing is not available and per-test costs amount to \$60–\$100. This may be too expensive for its widespread use in diagnosing TB, which typically affects socioeconomically disadvantaged groups. In contrast, per-sample reagent costs for in-house PCR-based TB diagnostic assays are less than \$5 per sample and this affordability may, in some settings, balance the procedural and logistical challenges of using in-house PCR assays.[19,20] Another commercial molecular method, the GenoType MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany), has made substantial contributions to the area of rapid diagnostics but still requires approximately 8 hours to complete the assay and additional training to ensure that results are generated and interpreted correctly.[21]

The aim of this study was to develop two multiplex fluorescence-based real-time PCR (qPCR) procedures to

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simultaneously identify the dominant mutations responsible for conferring rifampicin and isoniazid resistance in *M. tuberculosis*. The target mutations were: *katG*S315T and *inhA*-15C→T for isoniazid resistance, and *rpoB*S531L and *rpoB*H526Y for rifampicin resistance. Amplicons were identified based on melting-point curve analysis.

MATERIALS AND METHODS

Primer design. Primers were designed to detect four different mutations in three different genes by two simultaneous multiplex reactions. We targeted the amplicon melting temperature (T_M) as the first selection parameter, seeking appropriate primer sequences. Primers were designed so that resulting amplicons within one PCR assay would have T_Ms ranging from 76°C to 95°C, with > 1°C difference between peaks. Sequences of each gene were examined for features such as areas of high or low GC content, size, and identity among reported BLAST sequences for the target gene. These areas were analyzed by an oligonucleotide property calculator (Primer Premier 5.0, Premier Biosoft International, Palo Alto, CA), which uses the nearest-neighbor method to predict the amplicon's T_M. Once areas likely to produce amplicons with the desired T_M were selected, primers were designed using the Primer3 program (http://frodo.wi.mit.edu).

To detect both wild type (WT) and mutated sequences, we designed three oligonucleotides per mutation: a universal primer, a WT-detection primer, and a mutated-detection primer with the specific nucleotide in the 3′end. We added a short AT/GC–rich overhanging nucleotide sequence (flap) to the 5′ end in all the primers that recognized the mutants and in the primer that recognized the WT for *rpoB*531 to obtain sufficiently diverse T_Ms (Supplemental Table 1). The H37Rv reference strain sequence (NC_009525) was used as the WT genome.

In addition, the primers were analyzed with BLAST to analyze their specificity. Moreover, the Oligo Analyzer tool from the Integrated DNA Technologies website was used to identify the intra- and intercomplementarity of the primers. CLC Main Workbench (Waltham, MA) was used for predicting the T_M of the tailed primers and the tailed products.

Bacterial strains. The Mycobacteriology Unit of the Institute of Tropical Medicine, Antwerp (Belgium), provided 133 heat-inactivated bacterial suspensions. All strains were previously identified as *M. tuberculosis* and harboring at least one of the four mutations under investigation based on DNA sequencing. Phenotypic resistance to isoniazid and/or rifampicin was assessed using the Löwenstein–Jensen (LJ) proportion method.[22] Strains were identified as belonging to the *M. tuberculosis* complex by their susceptibility to para-nitrobenzoic acid on LJ medium[23] and their spoligotyping profiles.[24] Additionally, we tested 17 strains that were resistant to isoniazid and/or rifampicin but with other mutations in the target genes (Supplemental Table 2). In each qPCR run, we used the following strains as controls: H37Ra (WT, susceptible to isoniazid and rifampicin), TBDM2489 (mut *katG*S315T), LE371 (mut *rpoB*S531L), CSV4644 (mut *inhA*-15C→T), and ITM 04-2618 (mut *rpoB*H526Y). To assess the species specificity of the qPCR, we randomly selected 31 nontuberculous mycobacteria (NTM) reference strains belonging to 17 species from the public collection of mycobacterial cultures BCCM/ITM (Supplemental Table 3).

Real-time PCR conditions. qPCR was performed using a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany). Each multiplex PCR assay was performed in a 20 μL final reaction volume containing 2× SensiMix™ SYBR® No-ROX Kit (Bioline, Alphen aan den Rijn, The Netherlands), and the primers were used at a final concentration of 0.25–0.8 μM (Table 1). The amplification cycles consisted of an initial denaturation at 95°C for 10 minutes, 40 cycles of incubation at 95°C for 15 seconds, 63°C for 15 seconds, 72°C for 15 seconds, ending with a final extension at 75°C for 1 minute. After 40 cycles, a melting curve with a ramp rate of 0.02°C/second between 75°C and 96°C was generated. Melting peaks were automatically calculated by the software LightCycler 480 SW 1.5 (Roche Diagnostics, Rotkreuz, Switzerland) which, after subtracting background fluorescence from a set of water blanks, plotted the negative derivative of fluorescence with respect to temperature [−d(F)/dT versus T]. To control for cross-contamination and background noise, all runs included duplicate negative samples (no template control).

Analytical sensitivity. Genomic DNA (gDNA) was obtained from freshly grown LJ slants for the WT H37Ra strain by a simple heat inactivation method[25] and the four mutants (*katG*S315T, *rpoB*S531L, *inhA*-15C→T, and *rpoB*H526Y) by the cetyltrimethylammonium bromide method.[26] DNA concentration was measured with a NanoDrop 2000 UV-Vis

TABLE 1
Primers for multiplex real-time PCR

Gene	Location	Wild type	Mutant	Primers*	Final concentration (μM)	Amplicon size (base pairs)	Amplicon T_M (mean ± SD)
Multiplex 1							
katG				katG1-F	0.8	94	82.93 ± 0.10
	AA S315T	AGC	ACC	katG2-F	0.8	106	83.80 ± 0.15
				katG3-UR	0.8	–	–
rpoB				rpoB1-F	0.25	128	89.97 ± 0.07
	AA S531L	TCG	TTG	rpoB2-F	0.3	129	89.32 ± 0.16
				rpoB3-UR	0.3	–	–
Multiplex 2							
inhA				inhA3-UF	0.6	–	–
	Promotor −15	C	T	inhA1-R	0.6	106	85.19 ± 0.07
				inhA2-R	0.6	118	84.90 ± 0.08
rpoB				rpoB4-F	0.25	138	89.64 ± 0.06
	AA H526Y	CAC	TAC	rpoB5-F	0.25	141	89.49 ± 0.07
				rpoB6-UR	0.25	–	–

A = adenine; AA = amino acid; C = cytosine; F = forward; G = guanine; H = histidine; L = leucine; O = orientation; PCR = polymerase chain reaction; R = reverse; S = serine; SD = standard deviation; T = threonine; T = thymidine; T_M = melting temperature; Y = tyrosine.
*Primers sequences in Supplemental Table 2.

spectrophotometer Thermo Scientific (NanoDrop Technologies, Wilmington, DE). Ten-fold serial dilutions from 10^7 to 10^2 fg (approximately equivalent to 2×10^6–2×10^1 genome copies) were prepared in triplicate.[27]

Analysis. All laboratory procedures were performed by personnel who were blinded to sample details including the results of all other tests. The sensitivity and specificity of the qPCR to correctly identify our specific drug resistance–conferring mutations were calculated using 2 × 2 tables by comparing results to DNA sequencing that was considered the gold standard test. The 95% confidence intervals for sensitivity and specificity were calculated with the Wilson score method.[28]

Ethics. This laboratory research project used only strains from anonymous unlinked specimens and therefore was exempted from human subjects' research approval. None of the investigators have any conflict of interest in relation to this work.

RESULTS

"In silico" evaluation of designed primers. Supplemental Table 1 shows the results of parameters evaluated for the sets of designed primers for both multiplex assays. For all the primers, the ΔG (Gibbs energy) was far more negative for the correct target binding than for primer-dimers and hairpin structures.

Melting temperatures. After optimizing the primer sequences and concentrations to be used in each multiplex qPCR, the average T_Ms were between 82°C and 91°C for all amplicons (Table 1). The T_Ms of the amplicons were spaced such that the identification of multiple samples on a single graph is unambiguous. The spacing between peaks for each gene is shown in Figure 1. Remarkably little variation in T_M was observed among the different strains tested (T_M means ± standard deviations are given in Table 1). Also, the amplitudes of the melting curves were quite similar for all strains within each category.

Analytical sensitivity. To demonstrate the limit and range of the system to detect *M. tuberculosis* DNA, 10-fold serial dilutions containing 10^7–10^2 fg of gDNA (equivalent to 2×10^6–2×10^1 gDNA copies/reaction) were assayed in duplicate. The results were reported as threshold cycle numbers versus log of starting DNA quantities. Both assays detected

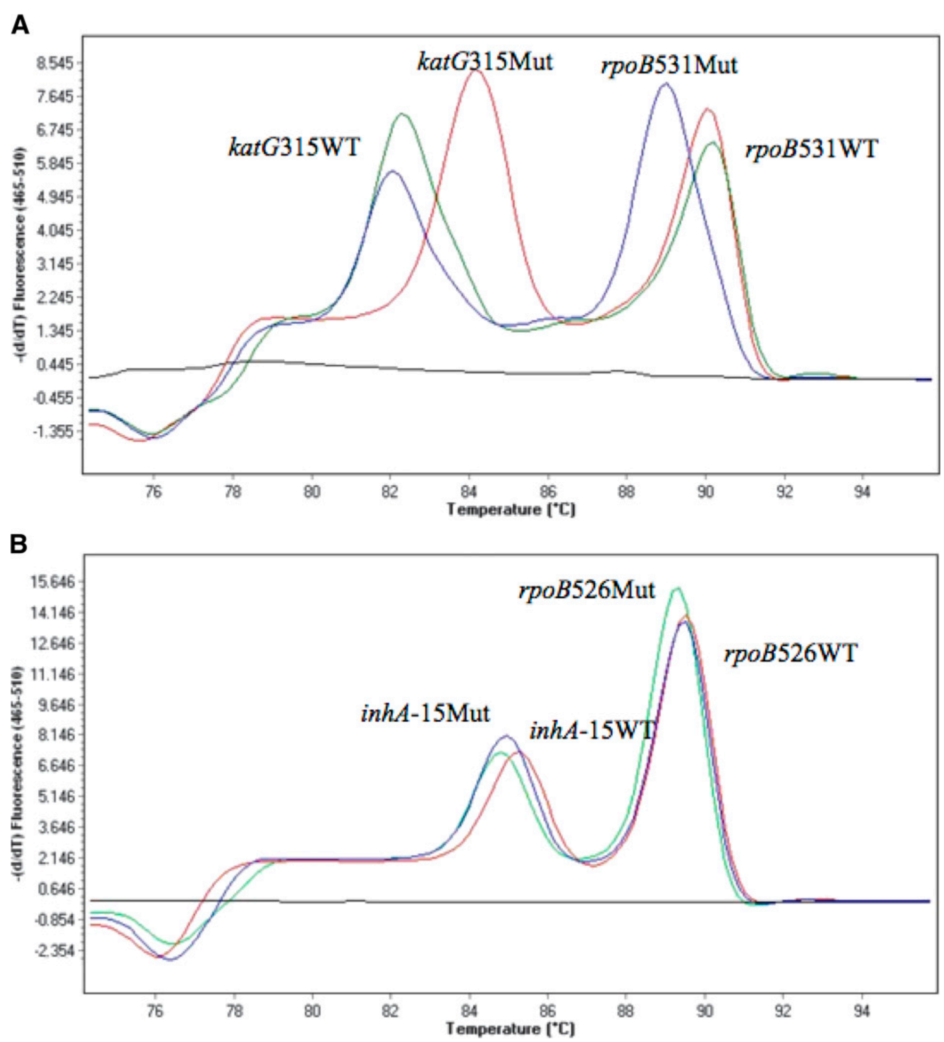

FIGURE 1. Melting temperature peaks graphic from wild type and mutant strains. (**A**) Peaks for Multiplex 1: H37Ra strain (red), mutant *katG*315 (green), and mutant *rpoB*531 (blue). (**B**) Peaks for Multiplex 2: H37Ra strain (red), mutant for *inhA*-15 (blue), and mutant for *inhA*-15 and *rpoB*526 (green). We included a negative control in all the runs (black).

the presence of WT and mutants in the range of 10^7–10^2 fg of gDNA. Therefore, the detection limit (standard curve method) was equivalent to about 20 bacilli/reaction.

The standard plot showed that the regression coefficient for Multiplex 1 was linear for WT, Mut *katG*315, and Mut *rpoB*531 (R^2 = 0.9998, 0.99847, and 0.99929, respectively) over a 10-log dilution range, and the reaction efficiencies were 90%, 95%, and 100% (Figure 2A–C, Supplemental Figure 1A–C). Similarly, the Multiplex 2 assay showed a linear regression coefficient for WT, Mut *inhA*-15, and Mut *rpoB*526 (R^2 = 0.9968, 0.9994, and 0.9999, respectively) over a 10-log dilution range, and the reaction efficiencies were 97%, 94%, and 90% (Figure 2D–F, Supplemental Figure 1D–F).

Application in heat-inactivated bacterial suspensions. We obtained valid qPCR results for 100% of *rpoB*531 (*N* = 148) and *inhA*-15 (*N* = 120), 98.6% for *katG*315 (*N* = 146), and 98.1% for *rpoB*526 (*N* = 104) of the tested samples. Two samples failed in Multiplex 1 for *kat*G (one WT strain and one mutant), whereas for Multiplex 2, we obtained invalid results for two samples with nontarget mutations. Overall, for the valid results, qPCR confirmed 100% of the susceptible strains (*N* = 50), and 100% of the *rpoB* and *katG* mutants, whereas the *inhA*-15T was detected in 95.5% (21/22) of the isolates. Combined, our assays correctly detected all MDR isolates tested. Among the 17 strains that presented other mutations in the target genes, our qPCR identified 3/7 strains resistant to rifampicin (samples 1, 4, and 7; Supplemental Table 3). Among the NTM samples, 11/31 (35%) gave a positive qPCR signal (data not shown), yet only 3/31 (9.7%) showed a T_M similar to the mutants; *Mycobacterium nonchromogenicum* for *rpoB*531, *Mycobacterium gastri* and *Mycobacterium gadium* for *katG*315. Hence, our systems showed sensitivities for *katG*315, *rpoB*531, *rpoB*526, and *inhA*-15 of 100%, 100%, 100%, and 96%, respectively; and specificities of 99%, 95%, 100%, and 100, respectively, compared with DNA sequencing (Table 2).

Speed and cost. The use of qPCR provided diagnostic information within 24 hours from the receipt of the sample. However, it can only be applied after TB confirmation with the TaqMan-based real-time PCR previously standardized in

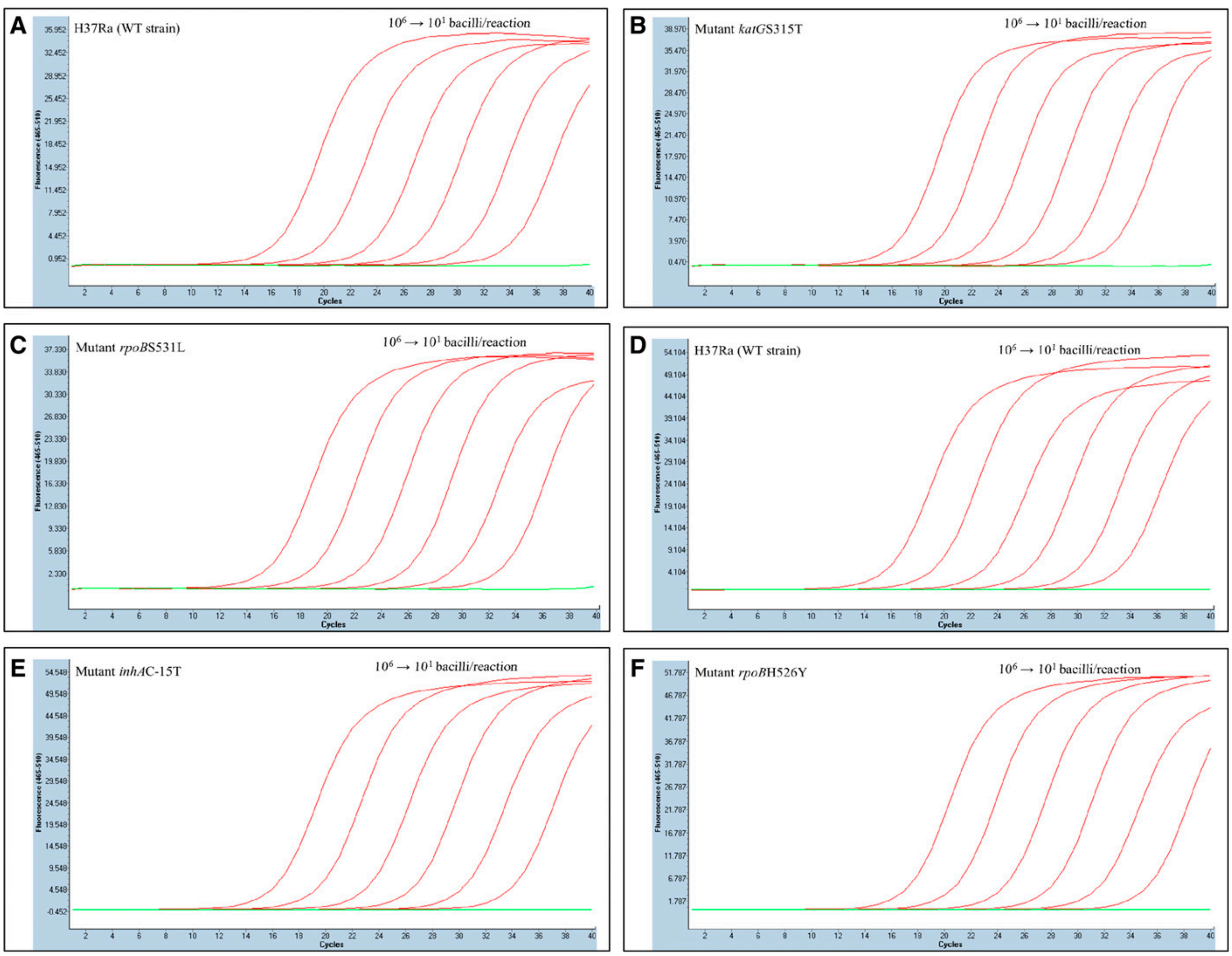

FIGURE 2. Limit of detection. Fluorescence from the real-time polymerase chain reaction products is plotted against the number of cycles. Ten-fold serial dilutions (red) from 10^7 to 10^1 fg (equivalent to 2×10^6–2×10^1 genomic DNA copies) were prepared for H37Ra and the mutants. Limit of detection of Multiplex 1 for: (**A**) H37Ra, (**B**) mutant *katG*315, and (**C**) mutant *rpoB*531. Limit of detection of Multiplex 2 for (**D**) H37Ra, (**E**) mutant *inhA*-15, and (**F**) mutant *rpoB*526. We included a negative control in all the runs (green).

TABLE 2
qPCR performance for the detection of isoniazid and/or rifampicin resistance in bacterial suspensions/reference strains

Genotype (sequencing*)	No. of samples	Phenotype (R/S)	qPCR			Sensitivity (95% CI)	Specificity (95% CI)
			Resistant	Susceptible	Failure/not defined		
Multiplex 1							
WT *katG* S315	50	S	0	49	1	100% (93–100%)	99% (94–100%)
Mut *katG* T315	50	R	49	0	1		
Other mutations†	17	†	0	17	0		
NTM	31	NA	1	30	0		
WT *rpoB* S531	50	S	0	50	0	100% (91–100%)	95% (88–98%)
Mut *rpoB* L531	50	R	50	0	0		
Other mutations†	17	†	3‡	14	0		
NTM	31	NA	2	29	0		
Multiplex 2							
WT *inhA* −15C	50	S	0	50	0	96% (75–99%)	100% (95–98%)
Mut *inhA* −15T	22	R	21	1	0		
Other mutations†	17	†	0	17	0		
NTM	31	NA	0	31	0		
WT *rpoB* H526	50	S	0	50	0	100% (60–100%)	100% (95–100%)
Mut rpoB Y526	8	R	8	0	0		
Other mutations†	17	†	0	15	2		
NTM	31	NA	0	31	0		

C = cytosine; CI = confidence interval; H = histidine; L = leucine; Mut = mutant; NA = not applicable; NTM = nontuberculous mycobacteria; qPCR = real-time polymerase chain reaction; S = serine; T = threonine; T = thymidine; WT = wild type; Y = tyrosine.
*Gold standard method.
†See Supplemental Table 3.
‡Codon S531P ($N = 1$), codon S533P ($N = 2$).

our laboratory[25] given the reaction with some NTM. Therefore, excluding the expense of the qPCR thermocycler device self and taking in account an additional qPCR for TB detection, the total reagent costs of our cascade qPCR would be approximately US$ 8.00 (Supplemental Table 4).

DISCUSSION

In this study, we have developed two multiplex qPCR assays that can be run simultaneously to detect isoniazid and rifampin resistance in *M. tuberculosis* by targeting the most common resistance-associated mutations in the *katG*, *inhA* promoter, and *rpoB* genes. The system was able to confirm 100% of the susceptible strains, and 100% of the *rpoB* and *katG* mutants, whereas the *inhA*-15T was detected in 95.5%.

Acquired drug resistance in *M. tuberculosis* is caused mainly by spontaneous mutations in chromosomal genes, producing the selection of resistant strains during suboptimal drug therapy. Several mutations have shown a range of association with isoniazid and rifampicin resistance. Although no single pleiotropic mutation has been found to cause specific resistance to a drug, there are some mutations that have shown an association with isoniazid and rifampicin. Resistance to isoniazid is a complex process that can involve several mutations in several genes; however, it has been shown that *katG*S315T and *inhA*C-15T are the most common mutations responsible for isoniazid resistance (64% and 19%, respectively).[29–31] In case of rifampicin resistance, mutations in a "hot-spot" region (rifampicin resistance–determining region) of 81 bp of *rpoB* have been found to be responsible in 96% of *M. tuberculosis* isolates.[32] The most common missense mutations associated were located at codons 531 and 526.[8,33,34] However, some studies have also reported other uncommon mutations inside and outside of the "hot-spot" region that were regularly or even systematically missed by standard, World Health Organization–endorsed DST methods.[35,36]

The most sensitive limit of detection theoretically possible is three copies per PCR reaction, assuming a Poisson distribution.[37] The analytical sensitivity is defined as the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure. Our results have shown that both assays could detect as little as 10^2 fg of DNA/reaction, which is equivalent to 20 bacilli/reaction. This compares to LJ culture, which detects 10–100 viable mycobacteria/mL of sample,[38] the GenoType MTBDR*plus* assay which detects 160 mycobacteria/mL,[39] and the GeneXpert System's MTB/RIF assay, which detects 131 colony-forming units/mL.[40]

Our results showed a sensitivity of 100% for rifampicin resistance associated to *rpoB* S531L and H526Y, in comparison to the 92% of Bactec MGIT 960 culture,[41] 100% of GenoType MTBC test (HAIN Lifescience),[42] and an overall sensitivity 98.9% for GeneXpert platform (Cepheid, Sunnyvale, CA).[43] In the case of isoniazid resistance, our results showed an overall sensitivity of 98% in comparison to 97% of Bactec MGIT 960 culture[44,45] and 100% of GenoType MTBC test (HAIN Lifescience) (Hillemann and others, 2007).[46]

Combined, our two multiplex assays reached a specificity of 96% for identifying mutants in *rpoB* in comparison to the Bactec MGIT 960 culture (100%), GenoType MTBC test (98%)[42] and the overall specificity of GeneXpert platform (99.8%) (Cepheid).[43] In the case of isoniazid resistance, our results confirmed all the mutants in *katG*; however, we could only confirm 95.5% (21/22) on mutants in *inhA*. Therefore, the overall specificity for isoniazid reached 99% in comparison to GenoType MTBC test (100%).

The target mutations selected for our assays explain 71–100% of isoniazid resistance, and 65% of rifampicin resistance.[36] There are other less common mutations within these genes, especially in *rpoB*; however, their frequencies were found to vary among *M. tuberculosis* isolates collected from different geographical locations.[47,48] In this study, we tested some isolates with these mutations to determine the performance of our assays (Supplemental Table 3). The three samples that were identified as rifampicin resistance might be explained by the presence of a mutation in the same nucleotide (S531P, *N* = 1) or adjacent to it (S533P, *N* = 2).

In the last decade, disease caused by NTM has gained attention, in part because of an assumed increase in its incidence.[49,50] The distribution of NTM species that are isolated from clinical samples differs strongly by region (Marras and others, 2002).[51] According to Hoefsloot and others,[52] the most frequently associated NTMs in South America are *Mycobacterium avium* and rapid-growing mycobacteria, for example, *Mycobacterium chelonae*.[53] In our study, only three of the 31 NTM amplified like a *M. tuberculosis* mutant that can be isolated from animals—that is, cattle, cervids, and deers[54]—and human samples, albeit rarely with clinical significance. Thus, both multiplex assays must be run after TB confirmation, for example, by the PCR previously standardized by our laboratory.[25] Abnormal or double melting peaks should alert for a possibly unnoticed presence of heteroresistance or mixture with NTM.

Although the prevalence of isoniazid resistance is much higher than that of rifampicin,[55] detection of isoniazid resistance has received lower priority because of its less pronounced clinical impact. However, a recent meta-analysis has suggested higher rates of failure/relapse and acquired resistance.[56,57] Mutations in *inhA* have been found more frequently associated with monoresistant strains,[58] whereas mutation S315T in *katG* occurs more frequently in MDR strains.[59] Therefore, if these multiplex assays are implemented into the workflow algorithm for detecting resistant strains, we would recommend to run both multiplex assays simultaneously after confirmation of TB.

Our protocols have some advantages. First, the use of a simplified DNA extraction using only heating and ethanol precipitation,[25] potentially facilitating implementation in resource-poor settings and avoiding the high cost of commercial kits for DNA isolation. Second, to avoid sole-source reagents and equipment that can be difficult to import, afford, and sustain in some settings, we used a SYBR Green real-time PCR assay and obtained sensitivity better than the much slower LJ culture. Third, excluding the expense of the qPCR thermocycler device self, the running cost (reagents and small materials) of this MTBC assay is ~US$7.00, lower than the Hain Assay (US$10.00 in Peru), and the GeneXpert MTB/RIF (US$10.00–$100 in different American countries). Fourth, they are less laborious and require less time to obtain results in comparison to the Hain Assay. Fifth, the qPCR can give information not only of the most common mutations associated to rifampicin resistance, but also to isoniazid resistance which remains undetected by GeneXpert MTB/RIF.

On the other hand, our assays also have some limitations. First, although the sequences used as the targets in these multiplex qPCRs are from highly conserved regions of the genes, a weakness of these or any multiplex assay is that new or less common mutations might fail to amplify with the primers described. Second, these assays also gave qPCR products for some of the NTM reference strains; therefore, they could only be applied after *M. tuberculosis* presence confirmation, for instance with our previous qPCR protocol for TB detection.[25]

In summary, the qPCR methods proposed in this study showed high specificity and sensitivity for the targeted mutations, short turn-around time, and relatively low cost that shows its potential for improving TB diagnosis and treatment. Therefore, further evaluation is needed to determine its diagnostic reliability in specimens in operational settings, and potential usefulness for routine clinical practice in settings with qPCR facilities.

Received February 16, 2016. Accepted for publication August 25, 2016.

Published online October 24, 2016.

Note: Supplemental tables and figure appear at www.ajtmh.org.

Acknowledgments: We thank our colleagues from Universidad Peruana Cayetano Heredia: Patricia Sheen, Mirko Zimic, and Patricia Fuentes for providing the primers used in this study, and David Requena for his assistance during the primer design phase. We thank our colleague David Durand from the Molecular Epidemiology Unit for his assistance with operating the LightCycler® 480 Real-Time PCR System. We also thank Maren Diels from the ITM, Antwerp (Belgium), for preparing the strains.

Financial support: This study was funded by the Belgian Directorate-General for Development Cooperation (DGDC) through an institutional collaboration between the Institute of Tropical Medicine in Antwerp, Belgium, and the Instituto de Medicina Tropical Alexander von Humboldt in Lima, Peru.

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