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Pyrosequencing for Rapid Detection of Extensively Drug-Resistant *Mycobacterium tuberculosis* in Clinical Isolates and Clinical Specimens

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Treating extensively drug-resistant (XDR) tuberculosis (TB) is a serious challenge. Culture-based drug susceptibility testing (DST) may take 4 weeks or longer from specimen collection to the availability of results. We developed a pyrosequencing (PSQ) assay including eight subassays for the rapid identification of *Mycobacterium tuberculosis* complex (MTBC) and concurrent detection of mutations associated with resistance to drugs defining XDR TB. The entire procedure, from DNA extraction to the availability of results, was accomplished within 6 h. The assay was validated for testing clinical isolates and clinical specimens, which improves the turnaround time for molecular DST and maximizes the benefit of using molecular testing. A total of 130 clinical isolates and 129 clinical specimens were studied. The correlations between the PSQ results and the phenotypic DST results were 94.3% for isoniazid, 98.7% for rifampin, 97.6% for quinolones (ofloxacin, levofloxacin, or moxifloxacin), 99.2% for amikacin, 99.2% for capreomycin, and 96.4% for kanamycin. For testing clinical specimens, the PSQ assay yielded a 98.4% sensitivity for detecting MTBC and a 95.8% sensitivity for generating complete sequencing results from all subassays. The PSQ assay was able to rapidly and accurately detect drug resistance mutations with the sequence information provided, which allows further study of the association of drug resistance or susceptibility with each mutation and the accumulation of such knowledge for future interpretation of results. Thus, reporting of false resistance for mutations known not to confer resistance can be prevented, which is a significant benefit of the assay over existing molecular diagnostic methods endorsed by the World Health Organization.

Regional increases in the prevalence of tuberculosis (TB) with drug resistance and a broad distribution of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB (1, 2) may reverse recent gains in global TB control (1). Molecular detection of mutations associated with drug resistance has facilitated rapid detection of drug resistance in the *Mycobacterium tuberculosis* complex (MTBC) (3–7), and the use of such molecular tools has become increasingly important in TB control and TB patient management (5, 7–10). Recognizing the advantages and disadvantages of various molecular methods will help in the selection of optimal methods for improved prediction of drug resistance in MTBC, which is critical in advancing molecular diagnostic approaches for this defined purpose.

In 2003, the Microbial Diseases Laboratory (MDL) at the California Department of Public Health developed a real-time, probe-based assay using molecular beacons for screening for MDR TB (11). Remarkable improvements in turnaround times for predicting resistance to isoniazid (INH) and rifampin (RIF) and significant impacts on the management of MDR TB (9) were realized. However, several incidents of detection of *rpoB* mutations not conferring RIF resistance resulted in erroneous reporting of false RIF resistance. Other investigators also encountered problems with detection of false RIF resistance with commercial probe-based assays (12–15). One of these problems was rooted in a general assumption that the presence of any mutation in the core region of *rpoB* predicts RIF resistance. Assays that detect the presence of mutations without providing sequence information do not allow users to evaluate if mutations are indeed present or to recognize mutations that do not confer resistance. A false-resistant result may only be realized weeks later, when phenotypic drug susceptibility testing (DST) results become available. Such an error may incorrectly guide a clinician to withhold an effective drug and make unneeded changes to the regimen, which may have adverse impact on patients.

In seeking improvements over probe-based assays, we explored pyrosequencing (PSQ) technology (16), a rapid real-time method for sequencing small segments of genomic DNA that has shown success in detecting mutations associated with drug resistance (3, 14, 17–21). Here, we report the development of a PSQ assay that was validated for testing both clinical specimens and isolates for the detection of mutations associated with resistance to INH, RIF, fluoroquinolones (fQs), amikacin (AMK), capreomycin (CAP), and kanamycin (KAN). California, with the highest numbers of MDR TB cases in the United States (22), was an ideal location for evaluating this assay. We also investigated whether this assay could address the specificity shortfalls observed with probe-based assays.

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MATERIALS AND METHODS

Specimens. A total of 259 samples, including 130 clinical isolates and 129 clinical specimens, were studied. The 130 clinical isolates were selected from the DNA collection of MTBC isolates at the MDL. We included 93 resistant isolates and 37 pan-susceptible isolates. The resistant isolates were selected to include diverse drug resistance profiles, and the susceptible isolates were randomly selected. Their phenotypic drug susceptibility profiles were previously characterized using the MGIT 960 system or the agar proportion (AP) method. These isolates were received initially in either solid medium (Lowenstein-Jensen medium or Middlebrook medium) or liquid medium (MGIT, BacT/Alert-MP broth, or Middlebrook 7H9 broth), and DNA was extracted from the growth in the original medium. The 129 clinical specimens originated from county laboratories where specimens were processed by the standard *N*-acetyl-L-cysteine (NALC)-NaOH procedure (23), and smears for acid-fast bacilli (AFB) were evaluated before submission to the MDL. AFB smear-negative specimens were not accepted unless they had previously tested positive by a nucleic acid amplification test. The distributions of AFB smear positivity for these specimens were 1+ ($n = 23$), 2+ ($n = 30$), 3+ ($n = 32$), 4+ ($n = 38$), and negative ($n = 6$). While the most common specimen type was sputum ($n = 115$ [89.1%]), 14 specimens (10.9%) were of other specimen types, including bronchial alveolar lavage fluid ($n = 2$), bronchial brushing ($n = 1$), pleural fluid ($n = 2$), respiratory aspirate ($n = 1$), gastric mass ($n = 1$), lymph node ($n = 1$), neck tissue ($n = 1$), peritoneal tissue ($n = 1$), spinal abscess ($n = 1$), cerebrospinal fluid ($n = 1$), and wound ($n = 2$) specimens.

DNA extraction. The crude DNA was extracted by heating cell suspensions at 95°C in a water bath as described previously (11), with two modifications. The cell pellets from the clinical specimens were resuspended in 100 μl of molecular biology-grade water, and the heating duration was extended to 25 min. DNA extracts from solid medium or liquid medium were tested at 1:100 dilution. If initial testing was not successful due to low peak heights, then the DNA was retested without dilution. DNA extracts from clinical specimens were tested undiluted. If DNA samples were not tested within 24 h, they were stored at −20°C.

Pyrosequencing assay and molecular targets. The PSQ assay involves three essential parts, namely, amplification of gene segments by PCR, capture of biotinylated single-stranded DNA on streptavidin-Sepharose beads, and sequencing with the PSQ technology (16). Our PSQ assay included eight subassays: one for identification of MTBC and seven for detection of drug resistance mutations. The molecular target of IS*6110* was for identification of MTBC. For INH, we chose three loci, i.e., *katG*, the *inhA* promoter, and the *ahpC* promoter. Note that mutations in the *ahpC* promoter may be compensatory for mutations in *katG*, which cause deficient catalase activities and are indirectly associated with INH resistance (24–26). The molecular targets for RIF, fQs and the three injectable drugs are the RIF resistance-determining region (RRDR) of *rpoB*, the quinolone resistance-determining region (QRDR) of *gyrA*, and *rrs*, respectively. The PCR primers and the sequencing primers are listed in Table 1. The PCR master mixes were prepared with the HotStarTaq kit and deoxynucleoside triphosphate (dNTP) mixtures (Qiagen, Valencia, CA). For each subassay, the PCR mixture included 2.5 μl of DNA and 22.5 μl of a PCR master mix specific for the subassay (see Table S1 in the supplemental material), which contained 1× PCR buffer, 2.5 mM $MgCl_2$, 0.96 mM dNTP mixture, 1× Q-solution, 0.5 μM each primer, and 1 U of HotStarTaq. The amplification parameters included initial activation of the HotStarTaq at 95°C for 15 min, 50 cycles of amplification at 94°C for 15 s, 60°C for 30 s, and 72°C for 20 s, and final extension at 72°C for 5 min. All subassays were tested simultaneously under the same PCR conditions. PSQ was performed using PyroMark Q96 (5 by 96) reagents with the sequence analysis mode of the PyroMark Q96 ID system (Qiagen, Valencia, CA). The dNTP dispensation order for each subassay was GCTCAC GTCAGTAGCACAC for IS*6110*, TGCTGCTGTGATC for *katG*, TGACA GTCGTATCGATCAGTCG for *inhA*, CTACTAGTCAGTGACGTGA GTCG for *ahpC*, TGTCACAGCATGCTGACGCGTACGTCAGTAGTAC GCAGACAGCGCTG for *rpoB1*, CTCGTCGACTGAGCAGCGTACGAC TGTCAGTGCGCTA for *rpoB2*, TCTGCGACGCTAGTCGATCTACTG ATCAGC for *gyrA*, and 4 repeats of TCGA for *rrs*. The sequence produced by the PyroMark Q96 ID was aligned against a library containing wild-type sequences and mutant sequences with IdentiFire software (Qiagen, Valencia, CA), to generate a PSQ report.

Assay specificity for MTBC. We evaluated the specificity of each subassay for MTBC by testing eight ATCC strains of nontuberculous mycobacteria (NTM) and 28 well-characterized clinical NTM strains from our archived collection. The ATCC strains were *Mycobacterium abscessus* (ATCC 19977), *Mycobacterium avium* (ATCC 25291), *Mycobacterium chelonae* (ATCC 35752), *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium gordonae* (ATCC 14470), *Mycobacterium intracellulare* (ATCC 13950), *Mycobacterium kansasii* (ATCC 12478), and *Mycobacterium scrofulaceum* (ATCC 19981). The clinical strains included *M. abscessus*, *Mycobacterium asiaticum*, *M. avium* complex, *Mycobacterium bohemicum*, *Mycobacterium celatum*, *M. chelonae*, *Mycobacterium elephantis*, *M. fortuitum*, *Mycobacterium gilvum*, *M. gordonae*, *Mycobacterium hecke-shornense*, *Mycobacterium holsaticum*, *Mycobacterium interjectum*, *M. kansasii*, *Mycobacterium kubicae*, *Mycobacterium lentiflavum*, *Mycobacterium margeritense*, *Mycobacterium marinum*, *Mycobacterium mucogenicum*, *Mycobacterium nebraskense*, *Mycobacterium obuense*, *Mycobacterium paraffinicum*, *Mycobacterium parascrofulaceum*, *Mycobacterium peregrinum*, *Mycobacterium simiae*, *Mycobacterium szulgai*, *Mycobacterium terrae*, and *Mycobacterium xenopi*.

Detection sensitivity. We evaluated the detection sensitivity of each subassay by testing serial dilutions of purified and quantitated DNA extracted from a reference strain of *Mycobacterium tuberculosis*, H37Rv (ATCC 27294). Ten-fold serial dilutions from 1 ng/μl to 100 fg/μl and 2-fold dilutions from 100 fg/μl to 25 fg/μl were made in molecular biology-grade water, followed by PSQ analysis. In addition, we studied the detection sensitivity in relation to smear positivity and CFU by seeding eight MTBC strains possessing various drug resistance mutations in smear- and culture-negative clinical specimens (sediments) processed by the NALC-NaOH procedure. Three cell suspensions of each strain, equivalent to 10^{-2}, 10^{-3}, and 10^{-4} dilutions of a 0.5 McFarland standard, were prepared in molecular biology-grade water, followed by 1:10 dilution with pooled smear- and culture-negative sediments to yield final concentrations equivalent to 10^{-3}, 10^{-4}, and 10^{-5} dilutions of a 0.5 McFarland standard. A total of 24 seeded specimens were prepared, and three tests were performed with each specimen, i.e., PSQ (0.5 ml), CFU analysis (0.1 ml onto each of two 7H10 plates), and an AFB smear with auramine-rhodamine staining.

Sanger sequencing and PSQ. Of the isolates included in this study, a total of 39 isolates were previously requested by TB clinicians for submission to the Reference Laboratory of the Division of TB Elimination Laboratory Branch at the Centers for Disease Control and Prevention (CDC) for molecular detection of drug resistance (MDDR) (6, 27), where Sanger sequencing was performed. The results of PSQ were compared with those of the Sanger sequencing to determine the accuracy of the PSQ results.

Reference method and data analysis. To analyze the association of mutations with phenotypic drug resistance, we compared the PSQ results with the DST results obtained previously with the MGIT 960 system or the agar proportion (AP) method. DST with primary drugs was performed when MTBC isolates were available, while DST with second-line drugs was performed for most isolates that were resistant to primary drugs. The drug concentrations tested with the MGIT 960 system for INH, RIF, ofloxacin (OFX), levofloxacin (LVX), moxifloxacin, AMK, and CAP were 0.1, 1.0, 2.0, 1.5, 0.25, 1.5, and 3.0 μg/ml, respectively (28, 29). The concentrations used in this study for the three quinolones are equivalent (29), and usually only one of the fQs was tested. KAN was not tested with the MGIT 960 system, because the critical concentration for KAN has not been well established. The drug concentrations tested with the AP method for INH, RIF, OFX, AMK, CAP, and KAN were 0.2, 1.0, 2.0, 4.0, 10, and 5.0 μg/ml, respectively (29). When there were discrepancies, PSQ was

TABLE 1 Primer sequences

Complex or drug	Locus and primers	Sequence	Amplicon size (bp)	Reference or source
MTBC	IS*6110*		110	
	Forward	Biotin-CCGCCAACTACGGTGTTTA		This study
	Reverse	CAGGCCGAGTTTGGTCAT		This study
	Sequencing	GGCCACCTCGATGCC		This study
INH	*katG*		67	
	Forward	Biotin-CGGAACCGGTAAGGACGC		This study
	Reverse	CCATTTCGTCGGGGTGTTC		This study
	Sequencing	TCCATACGACCTCGAT		This study
	inhA promoter		74	
	Forward	Biotin-ACGCTCGTGGACATACCG		This study
	Reverse	CAGTGGCTGTGGCAGTCA		This study
	Sequencing	TGTGGCAGTCACCCC		This study
	ahpC		104	
	Forward	TCCTCATCATCAAAGCGGACAAT		This study
	Reverse	Biotin-CGATGCCGATAAATATGGTGTGAT		This study
	Sequencing	CATTTGGTTGCGACAT		This study
RIF[a]	*rpoB*		182	
	Forward primer	TTTCGATCACACCGCAGACGTT		14
	Reverse primer	Biotin-AAAGGCACGCTCACGTGACAGAC		14 (modified in this study)
	Sequencing, for codons 507–521	GCGATCAAGGAGTTCTTC		This study
	Sequencing, for codons 522–533	CAGAACAACCCGCTG		This study
fQ	*gyrA*		225	
	Forward	AATGTTCGATTCCGGCTTCC		3
	Reverse	Biotin-CGGGCTTCGGTGTACCTCAT		3
	Sequencing	CAACTACCACCCGCA		This study
AMK, CAP, KAN	*rrs*		180	
	Forward	TAAAGCCGGTCTCAGTTCGGAA[b]C		This study
	Reverse	Biotin-CAGCTCCCTCCCGAGGGTTA		This study
	Sequencing	CTTGTACACACCGCC		This study

[a] For *rpoB*, a forward primer of GGAGGCGATCACACCGCAGACGTT and a reverse primer of biotin-CCTCCAGCCCGGCACGCTCACGT were also used in this study, but they work well only for the first segment (codons 507 to 521). The primers listed work for both segments.
[b] A was substituted for T in the natural sequence to improve specificity.

repeated; if isolates were available, phenotypic DST was also repeated. Performance characteristics were evaluated for the correlation between the PSQ assay and phenotypic DST, and the sensitivity and specificity of the PSQ assay for detecting resistance to each drug were determined. These analyses were presented in two ways, (i) based on the current practice applying a general assumption that the presence of a mutation predicts drug resistance and (ii) based on knowledge of the association of a mutation with phenotypic drug susceptibility or resistance.

RESULTS

Assay specificity for MTBC. For the 28 NTM species evaluated, the PSQ assay demonstrated 100% specificities for MTBC in all except the *rrs* subassay. Although the forward primer for *rrs* was designed with a substituted base to increase specificity, only 14 (50%) of the 28 species yielded no sequences and demonstrated specificity for MTBC. The other 14 NTM species generated sequences identical to that of MTBC in the 10-bp segment. These 14 species were *M. asiaticum*, *M. avium* complex, *M. bohemicum*, *M. elephantis*, *M. fortuitum*, *M. gordonae*, *M. interjectum*, *M. kansasii*, *M. margeritense*, *M. marinum*, *M. nebraskense*, *M. parascrofulaceum*, *M. scrofulaceum*, and *M. szulgai*. For *rpoB*, the segment could be amplified in most species, but the sequences were shorter than and distinctly different from that of MTBC. Therefore, the specificity of the two *rpoB* subassays for MTBC was still 100%.

Detection sensitivity. The detection sensitivity was assessed by testing serial dilutions of purified and quantitated DNA. The PSQ assay demonstrated 100% sensitivities in all subassays at 1 pg/μl. We further tested DNA at 100 fg/μl and 50 fg/μl in duplicate when PCR master mixes were prepared. Over a 5-month period, 10 batches of PCR master mixes were prepared using four different lots of HotStarTaq kits, and PSQ was performed with five different lots of PyroMark Q96 reagents. The detection sensitivity ranged from 85% to 100% for 100 fg/μl and from 75% to 100% for 50 fg/μl (see Table S2 in the supplemental material). Another assessment of detection sensitivity in relation to smear positivity and CFU was performed with 24 seeded specimens. Cell concentrations equivalent to 10^{-3}, 10^{-4}, and 10^{-5} dilutions of a 0.5 McFarland standard yielded smear results of 1+, doubtful/borderline (1 to 3 AFB per 100 fields at ×400 magnification), and negative, respectively. The specimens with positive smear results yielded 100% sensitivity and correct sequences in each subassay. The smear-negative specimens yielded 80 to 240 CFU/ml and 100% sensitivity (8/8 specimens) in the IS*6110*, *inhA*, and *rpoB* subas-

TABLE 2 Summary of PSQ results from clinical isolates and clinical specimens

PSQ result (no.)	Phenotypic DST result (no. of samples)[b]						Accuracy analysis results[a] (%)		
	Clinical isolates		Clinical specimens		Combined				
	R	S	R	S	R	S	Correlation	Sensitivity	Specificity
INH (245)									
Mutations detected	78	0	21	0	99	0	94.3	87.6	100
No mutations	10	42	4	90	14	132			
RIF (237)									
Mutations detected	67	3	12	3	79	6[c]	98.7[c]	96.3	100[c]
No mutations	1	55	2	94	3	149			
fQs (125)[d]									
Mutations detected	17	0	3	0	20	0	97.6	87.0	100
No mutations	2	79	1	23	3	102			
AMK (120)									
Mutations detected	14	1	1	0	15	1	99.2	100	99.0
No mutations	0	79	0	25	0	104			
CAP (119)									
Mutations detected	14	1	1	0	15	1	99.2	100	99.0
No mutations	0	79	0	24	0	103			
KAN (55)									
Mutations detected	11	0	1	0	12	0	96.4	85.7	100
No mutations	2	28	0	13	2	41			

[a] The accuracy analysis was performed with combined data from clinical isolates and clinical specimens. The accuracy analysis for the clinical isolates only is presented in Table S4 in the supplemental material and that for the clinical specimens in Table S5 in the supplemental material.
[b] R, resistant; S, susceptible.
[c] The analysis for RIF was based on known associations of specific *rpoB* mutations with RIF resistance or susceptibility. The mutations not conferring resistance were CAC526AAC ($n = 2$) and TTC514TTT ($n = 4$). The analysis excluded samples with *rpoB* mutations with unknown or inconsistent associations with RIF resistance, i.e., CTG511CCG, GAC516TTC, CAC526TGC, CAC526AGC, CAC526TCC, and CTG533CCG. For an analysis based on the general assumption that any *rpoB* mutation is a predictor of RIF resistance, the correlation and specificity would be 96.2% and 96.1%, respectively.
[d] An isolate was classified as fQ resistant when it tested resistant to one of the fQs at its test concentration, i.e., 2 μg/ml for ofloxacin, 1.5 μg/ml for levofloxacin, and 0.25 μg/ml for moxifloxacin.

says, 87.5% (7/8 specimens) in the *katG*, *ahpC*, and *rrs* subassays, and 75% (6/8 specimens) in the *gyrA* subassay (see Table S2 in the supplemental material).

Comparison of PSQ and Sanger sequencing results. The results of PSQ and Sanger sequencing were available for comparison for 39 isolates. Five molecular targets (*katG*, *inhA*, *rpoB*, *gyrA*, and *rrs*) overlapped between the CDC MDDR and our PSQ panels. A total of 195 results were generated, with a correlation of 99.0% (193/195 results) between the two methods. One INH-resistant isolate had a mutation in *katG* (ACT380ATT) that was out of the detectable range of our *katG* subassay but was detected by Sanger sequencing. Another INH-resistant isolate, with no mutations detected in *katG* or the *inhA* promoter by both methods, had a mutation in *ahpC* (G to A at position −10) detected by our *ahpC* subassay. Because the MDDR panel did not include the *ahpC* locus, the mutation was not detected. If the two sets of data are examined within the detectable ranges of each method, then these two results should not be counted as true discrepancies; thus, 100% correlation was realized. Table S3 in the supplemental material shows 69 mutations detected by the two methods in concordance. There were several rarely encountered mutations, such as CAC526AGC, CAC526TGC, CAC526TCC, and CAC526AAC in *rpoB*, GGC88TGC and GCG90AAG in *gyrA*, and dual mutations of CAC526TCC and AAG527CGG in *rpoB*. These findings demonstrated the fidelity of the PSQ assay in its ability to detect mutations.

PSQ performance in testing clinical isolates. The overall results and data analysis for combined clinical isolates and specimens are shown in Table 2, while Table S4 in the supplemental material shows the results and data analysis specifically for the clinical isolates. INH DST results were available for 130 isolates, of which 88 were resistant. The PSQ assay detected 82 mutations in 78 resistant isolates, with 88.6% sensitivity (78/88 isolates). Those mutations were found in *katG* ($n = 53$ [64.6%]), *inhA* ($n = 25$ [30.5%]), and *ahpC* ($n = 4$ [4.9%]); three isolates had mutations in both *katG* and *inhA*, and one isolate possessed mutations in both *katG* and *ahpC*. The specificity for predicting INH resistance was 100% (42/42 isolates), and the overall correlation between the PSQ and phenotypic DST results was 92.3% (120/130 isolates).

RIF DST results were available for 130 isolates; four isolates were excluded from analysis. One isolate had the GAC516TTC mutation with discrepant RIF results (resistant by the AP method but susceptible by the MGIT 960 system). Three isolates had mutations (CTG511CCG, CAC526TGC, and CAC526AGC) new to the MDL. We had not studied RIF MICs for these mutations prior to this study, although we were familiar with inconsistent RIF DST

results associated with the CTG511CCG mutation (14, 30). Therefore, we did not attempt to predict the association with RIF resistance for these mutations. Of the remaining 126 isolates, 68 were resistant and 58 were susceptible. Mutations in the RRDR were detected in 67 resistant isolates, with 98.5% sensitivity (67/68 isolates). Of the 58 susceptible isolates, mutations were detected in three isolates, i.e., CAC526AAC ($n = 2$) and TTC514TTT ($n = 1$, a synonymous mutation). When we analyzed our data with the assumption that the presence of any mutation in the RRDR predicts RIF resistance, the specificity was 94.8% (55/58 isolates) and the correlation was 96.8% (122/126 isolates). Synonymous mutations are not associated with RIF resistance. Previous isolates with the CAC526AAC mutation tested RIF susceptible with the MGIT 960 system at the MDL; we also found in another study that isolates with this mutation tested RIF susceptible with the AP method (6). Thus, with the availability of sequence results and the knowledge of the association of mutations with RIF phenotypic DST results from our past experience and from the literature, we correctly interpreted PSQ results, including mutations not conferring resistance. Therefore, the specificity for detecting RIF resistance was 100% (58/58 isolates) and the correlation was 99.2% (125/126 isolates).

DST results for fQs were available for 98 isolates. Isolates resistant to any of the three quinolones were classified as resistant. Of the 98 isolates, 19 were resistant. Mutations in the QRDR of *gyrA* were detected in 17 resistant isolates, yielding 89.5% sensitivity (17/19 isolates). The specificity for detecting resistance to fQs was 100% (79/79 isolates), and the correlation was 98.0% (96/98 isolates).

DST results for AMK were available for 94 isolates, of which 14 were resistant. The A1401G mutation in *rrs* was detected in all 14 resistant isolates and resulted in a 100% sensitivity. Due to a mutation (C1402T) detected in an AMK-susceptible isolate, the specificity was 98.8% (79/80 isolates) and the correlation was 98.9% (93/94 isolates). For CAP, DST results were available for 94 isolates, of which 14 were resistant. Mutations in *rrs* were detected in all 14 resistant isolates, which included the A1401G ($n = 13$) and C1402T ($n = 1$) mutations, yielding a 100% sensitivity. Because an isolate with the A1401G mutation tested CAP susceptible, the specificity was 98.8% (79/80 isolates) and the correlation was 98.9% (93/94 isolates). For KAN, DST results were available for 41 isolates, of which 13 were resistant. The A1401G mutation was detected in 11 KAN-resistant isolates, yielding 84.6% sensitivity (11/13 isolates). The specificity was 100% (28/28 isolates), and the correlation was 95.1% (39/41 isolates).

PSQ performance in testing clinical specimens. It is of great interest to know the detection sensitivity of the assay for testing clinical specimens. Of the 129 specimens, the rate of yielding complete sequence results was 89.1% (115/129 specimens). We found that four specimens grew NTM (*Mycobacterium avium* complex and *Mycobacterium fortuitum*), two specimens were contaminated, and one specimen yielded no growth. Of the remaining 122 specimens, the assay had a 98.4% sensitivity (120/122 isolates) for MTBC detection and a 95.8% sensitivity (115/120 isolates) for complete mutation detection. The five specimens with partial results failed to show amplification in one or two subassays, particularly in the *gyrA* subassay, although MTBC was identified.

The data for the clinical specimens are shown in Table 2, and the accuracy analyses of the sensitivity, specificity, and correlation of the PSQ and phenotypic DST results are shown in Table S5 in the supplemental material. For INH, PSQ results were available for 119 specimens; DST results were available for 115 specimens because of a lack of growth in four specimens. Among the 115 specimens, 25 were resistant, and mutations were detected in 21 specimens, yielding an 84.0% sensitivity (21/25 specimens). The mutations were found in *katG* ($n = 14$ [66.7%]), *inhA* ($n = 6$ [28.6%]), and *ahpC* ($n = 1$ [4.8%]). The specificity was 100% (90/90 specimens), and the correlation was 96.5% (111/115 specimens).

For RIF, the PSQ assay yielded valid results for 118 specimens; DST results were available for 114 specimens because of a lack of growth in four specimens. Three DST results were excluded from analysis. One specimen had the GAC516TTC mutation with inconsistent RIF results (resistant by the AP method but susceptible by the MGIT 960 system). Another specimen had the CTG533CCG mutation. Isolates with this mutation showed inconsistent RIF results, as revealed in published studies (6, 20, 30, 31), but our testing of previous isolates with this mutation showed a RIF MIC of 0.5 μg/ml with the MGIT 960 system, indicating RIF susceptibility. Because the number of isolates tested at the MDL was small, we categorized the association of this mutation with RIF resistance as uncertain. The third specimen had the CAC526TCC mutation, which was new to us, and we were unable to predict its association with RIF resistance. Of the remaining 111 specimens, 14 were resistant. Mutations in the RRDR were detected in 12 resistant specimens, yielding 85.7% sensitivity (12/14 specimens). Of the 97 specimens susceptible to RIF, no mutations were detected in 94, but a synonymous mutation, TTC514TTT, was detected in three specimens. If the accuracy analysis was based on the assumption that the presence of any mutation in the RRDR predicts RIF resistance, then the specificity would be 96.9% (94/97 specimens) and the correlation would be 95.5% (106/111 specimens). With the availability of sequence results and the established knowledge of the association of mutations with phenotypic DST results, we correctly interpreted the PSQ results. Therefore, the specificity for RIF was 100% (97/97 specimens), and the correlation was 98.2% (109/111 specimens).

For fQs, PSQ results were available for 115 specimens. DST for fQs was performed for 27 specimens, because only a few specimens showed resistance to primary drugs. Four specimens were resistant, with three containing mutations in the QRDR. The specificity was 100% (23/23 specimens), and the correlation was 96.3% (26/27 specimens).

For injectable drugs, PSQ results were available for 118 specimens. DST for injectable drugs was performed for a limited number of specimens, i.e., 26 for AMK, 25 for CAP, and 14 for KAN, because only a few specimens were resistant to primary drugs. We detected the A1401G mutation in *rrs* in one specimen, which tested resistant to the three injectable drugs. Since no discrepancies were found, it indicated 100% specificity and a 100% correlation.

Because few clinical specimens were resistant to fQs ($n = 4$) and injectable drugs ($n = 1$), sensitivity analyses for clinical specimens alone for those drugs would not be meaningful. Therefore, we present the accuracy analyses using the combined data for the clinical isolates and clinical specimens (Table 2). In summary, correlations with phenotypic DST results exceeded 94% for all drugs, ranging from 94.3% to 99.2%. Sensitivity in detecting resistance ranged from 85.7% to 100%. Specificity values were excellent, in the range of 99% to 100% for all drugs. In addition, all of the mutations detected in this study and their associations with

phenotypic DST results are shown in Table S6 in the supplemental material.

DISCUSSION

PSQ performs real-time sequencing of short DNA segments at a speed averaging 1 min per nucleotide. We adapted an existing platform (16) and developed an assay for rapid detection of XDR TB. The strengths of this study are as follows. (i) We included a target to identify MTBC, concurrent with drug resistance detection. This has not been done in most published PSQ studies, and it was an improvement over the recommendation for identification of MTBC prior to drug resistance detection made by Guo et al. in a meta-analysis publication (32). (ii) Our assay is sensitive enough to test clinical specimens, which significantly shortens the turnaround time for obtaining molecular DST results. (iii) We studied specificity for MTBC against NTM in each subassay. This is critical, especially for testing clinical specimens, when the presence of MTBC is unknown. (iv) All subassays were optimized to test simultaneously throughout the procedure, which increases the utility for clinical or public health laboratories and is a significant improvement over several published studies (3, 17, 21) requiring different testing conditions for different loci.

Our data showed that the specificity for MTBC was 100% in all but the *rrs* subassay, due to the highly conserved nature of the locus. Since the IS*6110* subassay has 100% specificity for MTBC with the highest sensitivity, it serves as a gatekeeper for validity checks of *rrs* results; the *rrs* results can be considered valid only when the IS*6110* subassay yields positive results for MTBC. Therefore, the impact of the decreased specificity of the *rrs* subassay is minimized.

The understanding of the genetic basis of INH resistance is incomplete. The sensitivity of detecting INH resistance mutations from *katG* and the *inhA* promoter was only 82.7%, as shown in our previous study (11). To increase the sensitivity, we added *ahpC* in our PSQ panel. In a study (31) conducted by Huang et al., 18 of 28 MDR TB isolates possessed *ahpC* mutations alone or in addition to *katG* mutations outside of codon 315. Detection of INH resistance from these isolates was missed by the MTBDR*plus* assay; this demonstrated the significance of adding the *ahpC* locus for detecting INH resistance. Our study found a rather small increase (4.5%) in sensitivity contributed by *ahpC*. However, any increase in the detection of INH resistance reduces ineffective INH treatment and prophylaxis and is welcomed by clinicians and public health authorities, especially when the specificity remains 100%.

RIF is a powerful drug for treating TB. The accuracy of molecular testing for RIF resistance is critical, since it may influence early clinical decisions. As shown in Table 2, mutations not conferring RIF resistance were detected. If we assume that any mutation in the RRDR is an indicator of RIF resistance, then the specificity would be 96.1%. Using a probe-based assay with this level of specificity in a country such as the United States, where the prevalence of RIF resistance (1%) is low, can be problematic because of low positive predictive values. Therefore, we recommend confirmation with a sequence-based method when a mutation is detected by a method that does not have the capability of providing the identity of the mutation. In addition to the uncertainty of *rpoB* mutations in association with RIF resistance, there is a similar issue for *rrs* with resistance to injectable drugs. This realization will likely extend to mutations associated with other classes of drugs as molecular assays for mutation detection are more widely implemented. The sequence information provided by PSQ or other sequence-based assays and the evolving knowledge of the MIC range associated with each mutation allow users to interpret and to report mutations properly. When novel mutations or mutations with uncertain correlation with drug resistance are detected, the prediction of their association with drug resistance should be deferred to phenotypic DST results. Thus, reporting of false resistance for mutations that are known not to confer resistance can be prevented.

The most frequent mutation in *rrs* associated with resistance to the injectable drugs is the A1401G mutation, followed by C1402T and G1484T mutations. Our assay can detect the A1401G and C1402T mutations, but it requires another set of primers to detect the G1484T mutation. Since the prevalence of the G1484T mutation is low and the resistance rate for the injectable drugs in California is also low, it may not be cost-effective to add a subassay for detection of this mutation. With a limited number of resistant isolates tested, our assay showed 100% sensitivity for detecting AMK and CAP resistance. However, like the data shown in a review article (33) and other studies (6, 34, 35), we predict that sensitivity is likely to decrease when more-diverse specimens resistant to AMK or CAP are tested. For KAN, the sensitivity was only 85%, which could be increased by testing the *eis* promoter, mutations in which have been shown to account for nearly 20% of KAN resistance in global clinical isolates (33).

A technical issue of the misreading of homopolymers should be noted. This phenomenon was discussed in previous publications (3, 36, 37) and was observed in our study as well. We noted that the most-frequent misreads occurred in codons 522 and 523 of *rpoB*, where 4 Gs may be read as 3 Gs by the software. The segment of codons 522 to 533 of *rpoB* is rather difficult to sequence, possibly because of the presence of several homopolymers, a high GC ratio, and perhaps unfavorable secondary/tertiary DNA structures at 28°C, the PSQ operating temperature. The end of this segment was codon 533 (CTG), which was followed by codon 534 (GGG). We left the very last dGTP out of the dNTP dispensation to avoid sequencing another 4 Gs at the end of this segment and to reduce the possibility of homopolymer misreading. When a double peak height for "C" is observed at the end of the sequence, it indicates the presence of the CTG533CCG mutation. Although this is not a commonly encountered mutation, retesting with the addition of guanine to the dNTP dispensation is recommended to confirm it. Alternatively, another set of primers (rB534BF2, biotin-CTGTCGGGGTTGACCCAC; rB534R2, ACG CTCACGTGACAGACCG) and a sequencing primer (rB534S2, TGACAGACCGCCGGG) were designed to sequence codon 534 to 527 of the reverse strand, which can also be used to confirm this mutation.

A recent report (38) used Illumina MiSeq, one of the next-generation sequencing (NGS) platforms, to perform whole-genome sequencing for rapid identification of MTBC, DST, and genotyping. The NGS platforms and the PSQ platform we used in this study have substantially different goals and approaches. Our PSQ assay focuses on detecting only the most prevalent mutations within a very short stretch of nucleotides (<50 bases). Test implementation and data analysis are simple; the information obtained is very useful but limited. In contrast, NGS allows interrogation of many genes with sequencing of much longer segments but requires sophisticated software to handle huge data sets. The current

NGS platforms need higher DNA concentrations (>50 ng), necessitating growth from cultures for testing. Although our PSQ assay has the advantage of being sensitive enough to test clinical specimens, it has a major limitation. It cannot be practically applied when mutations are spread widely over a gene, as is the case in *pncA*; thus, it is difficult to use for pyrazinamide resistance detection.

In conclusion, our data are comparable to the published data generated with various molecular methods (3, 4, 17, 31, 34, 35, 39), and they demonstrate that the PSQ assay is a rapid effective method for identifying MTBC and detecting drug resistance mutations from clinical specimens and isolates. The entire procedure, from extracting DNA to reporting results, can be accomplished within 6 h using a protocol that can be easily integrated into the workflow of diagnostic or public health laboratories. The estimated material cost per specimen for the assay, including all eight subassays from PCR to PSQ, is $60. The accuracy of sequencing by PSQ is comparable to that of Sanger sequencing, and the availability of sequence information enables users to study the association of drug MICs with each mutation. Once sufficient data associating specific mutations with MIC distributions have been accumulated, PSQ may become a useful tool for predicting the level of drug resistance. Such information may help guide treatment decisions before the availability of phenotypic DST results. This is a significant advantage of PSQ over probe-based methods and sets a higher standard for molecular detection of drug resistance. Based on the outcome of this study, a larger field trial of this assay is under way in India, Moldova, and South Africa. In addition, an evaluation of the impact of this assay on TB patient management is in progress in California.

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