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Predicting Extensively Drug-Resistant *Mycobacterium tuberculosis* Phenotypes with Genetic Mutations

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Molecular diagnostic methods based on the detection of mutations conferring drug resistance are promising technologies for rapidly detecting multidrug-/extensively drug-resistant tuberculosis (M/XDR TB), but large studies of mutations as markers of resistance are rare. The Global Consortium for Drug-Resistant TB Diagnostics analyzed 417 *Mycobacterium tuberculosis* isolates from multinational sites with a high prevalence of drug resistance to determine the sensitivities and specificities of mutations associated with M/XDR TB to inform the development of rapid diagnostic methods. We collected M/XDR TB isolates from regions of high TB burden in India, Moldova, the Philippines, and South Africa. The isolates underwent standardized phenotypic drug susceptibility testing (DST) to isoniazid (INH), rifampin (RIF), moxifloxacin (MOX), ofloxacin (OFX), amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) using MGIT 960 and WHO-recommended critical concentrations. Eight genes (*katG*, *inhA*, *rpoB*, *gyrA*, *gyrB*, *rrs*, *eis*, and *tlyA*) were sequenced using Sanger sequencing. Three hundred seventy isolates were INH^r, 356 were RIF^r, 292 were MOX^r/OFX^r, 230 were AMK^r, 219 were CAP^r, and 286 were KAN^r. Four single nucleotide polymorphisms (SNPs) in *katG*/*inhA* had a combined sensitivity of 96% and specificities of 97 to 100% for the detection of INH^r. Eleven SNPs in *rpoB* had a combined sensitivity of 98% for RIF^r. Eight SNPs in *gyrA* codons 88 to 94 had sensitivities of 90% for MOX^r/OFX^r. The *rrs* 1401/1484 SNPs had 89 to 90% sensitivity for detecting AMK^r/CAP^r but 71% sensitivity for KAN^r. Adding *eis* promoter SNPs increased the sensitivity to 93% for detecting AMK^r and to 91% for detecting KAN^r. Approximately 30 SNPs in six genes predicted clinically relevant XDR-TB phenotypes with 90 to 98% sensitivity and almost 100% specificity.

While tuberculosis (TB) incidence continues to decline globally, the prevalence of drug-resistant TB (DR TB) has continued to increase (1). The World Health Organization (WHO) reported an estimated 450,000 new cases of multidrug-resistant TB (MDR TB) worldwide in 2012, of which 9.6% were also extensively drug-resistant TB (XDR TB) (2). The increasing prevalence of M/XDR TB likely is in large part due to the fact that <25% of new MDR-TB cases are being diagnosed (2), leaving >250,000 patients per year with undiagnosed and untreated MDR TB to transmit primary DR TB in their communities.

One of the principle reasons why the diagnosis of M/XDR TB lags so significantly behind disease incidence is that the primary means of diagnosing clinical DR TB currently requires phenotypic drug susceptibility testing (DST) based on slowly growing mycobacterial cultures. Diagnosing MDR TB, which involves resistance to isoniazid (INH) and rifampin (RIF), and XDR TB, which additionally involves resistance to fluoroquinolones (FQ) and at least one of the three injectable anti-TB drugs (amikacin [AMK], kanamycin [KAN], and capreomycin [CAP]), requires completing DST with six or more drugs. Under the best conditions, this procedure takes several weeks to months, by which time many patients, especially those with HIV/XDR TB, have already died (3). Furthermore, studies of newly treated TB patients suggest that while correctly diagnosed and adequately treated patients stop transmitting TB very quickly, patients with undetected and inadequately treated DR TB continue to transmit not just TB but DR TB (4, 5), resulting in significant risks to the public.

Molecular diagnostic methods, such as line probe assays and GeneXpert, which rely on the rapid detection of mutations associated with drug resistance rather than phenotypic growth, have begun to significantly alter the M/XDR-TB diagnostic landscape (6–8). However, as molecular diagnostics (which detect resistance-conferring mutations) are indirect measures of phenotypic resistance, and the genetics of drug resistance are not yet completely understood, these diagnostics have introduced new complexities into the clinical evaluation of drug resistance in TB patients. The WHO has approved the use of line probe assays for MDR-TB diagnosis, but not as "a complete replacement for con-

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ventional culture and DST" (9), and they have yet to recommend the use of molecular diagnostics for XDR-TB (10).

If molecular diagnostics are to replace or complement phenotypic DST for diagnosing XDR TB in the near future, we need to demonstrate with strong empirical evidence that resistance-associated mutations are precise and quantifiable predictors of globally prevalent phenotypic resistance that can be used in rapid clinical decision making. To further this goal, the Global Consortium for Drug-Resistant TB Diagnostics (GCDD) conducted a detailed analysis of the sensitivities and specificities of the major mutations known to be associated with drug resistance in clinical TB isolates from regions of high M/XDR-TB burden in India, Moldova, the Philippines, and South Africa. In this study, we describe the dominant single nucleotide polymorphisms (SNPs) associated with resistance to drugs used to treat XDR TB and how well those SNPs predict phenotypic resistance as defined by WHO MGIT 960 DST standards.

MATERIALS AND METHODS

Sample collection strategy. The goal of this study was to obtain a large collection of clinical TB isolates that maximized the diversity of M/XDR-TB phenotypes from different global regions with a high burden of M/XDR TB. In order to develop an idea of the global distribution of mutations associated with M/XDR TB, we identified four sites with very different clinical and epidemiological characteristics. We focused our collection on sites in India, Moldova, the Philippines, and South Africa. The goal was to collect 100 to 150 clinical M/XDR-TB strains and a selection of pansusceptible strains from each site.

Sample representativeness. Our sample was drawn from TB repositories at (i) Hinduja National Hospital (PDHNH) in Mumbai, India, representing Mumbai strains, (ii) the Phthisiopneumology Institute (PPI) in Chisinau, the central unit of the National TB Control in Moldova, representing national TB strains in Moldova, (iii) the Tropical Disease Foundation (TDF) in Manila, Philippines, representing Manila strains, and (iv) the National Health Laboratory Service of South Africa (NHLS) in Johannesburg, representing a national archive of South African TB strains. All strains were considered independent in that only one strain was collected per TB patient from each repository.

Isolate selection. We selected all M/XDR-TB isolates in a particular repository if there were <100 strains available. In order to maximize phenotypic diversity if there were >100 M/XDR-TB strains available, we selected the strains with the most diverse DST profiles first and randomly selected strains with identical DST profiles until ~100 were selected. We randomly selected pansusceptible *Mycobacterium tuberculosis* strains from each site. All selected isolates were transferred to the University of California San Diego (UCSD), for standardized DST and sequencing.

Phenotypic drug susceptibility testing. Each isolate was subjected to standardized DST to INH, RIF, moxifloxacin (MOX), ofloxacin (OFX), AMK, KAN, and CAP on the mycobacterial growth indicator tube (MGIT) 960 platform, using the EpiCenter software (BD Diagnostic Systems, Franklin Lakes, NJ, USA), according to the standard manufacturer protocols (11) and the WHO-recommended critical concentrations for MGIT-based DST (see Table 2 for critical concentrations used) (12). As there were no published WHO-recommended critical concentrations for KAN DST by MGIT 960 at the time of the study, we used 2.5 mg/liter based on the literature (13).

Selecting genes associated with drug resistance for sequencing. Between 2010 and 2012, our group conducted comprehensive systematic reviews of the English-language literature to find studies with strong evidence relating mutations to phenotypic resistance to the drugs INH, RIF, MOX, OFX, AMK, KAN, or CAP. Based on our reviews (14) and those of others (15–17), we determined that mutations in eight genes were associated with the majority of the observed phenotypic resistance to these drugs globally. We sequenced all or part of each of these genes in every isolate, depending on evidence supporting the clustering of mutations in a particular gene. If there was no strong evidence for mutation clustering (e.g., a mutation in *tlyA* only), we sequenced the whole gene. If there was strong evidence of clustering (e.g., mutations in *katG*, *rpoB*, *gyrA*, and *rrs*), we sequenced ~200 to 300 bp of the gene surrounding the regions where mutations were reported to be clustered. The promoters *inhA* and *eis* were sequenced in their entirety.

DNA extraction and PCR. DNA extraction and amplification of the gene fragments for Sanger sequencing were performed using standard methodologies (18). Briefly, isolates were grown on Löwenstein-Jensen medium, killed by exposure to ethanol and heat, and lysed. DNA was extracted using NaCl, cetyltrimethylammonium bromide (CTAB), and chloroform-isoamyl alcohol. The gene fragments were amplified using real-time PCR, with the exception of *tlyA*, which was amplified in its entirety using conventional PCR because of the large amplicon size. See Table 1 for the primer and PCR product details.

Sanger sequencing. Sanger sequencing of PCR fragments was performed by the Center for Advanced Studies of Genomics, Proteomics, and Bioinformatics at the University of Hawaii, Manoa, using an Applied Biosystems 3730xl analyzer with the BigDye Terminator version 3.1 cycle sequencing kit and standard manufacturer protocols. Forward primers were used for sense sequencing of the product, with the exception of *tlyA*, which was assembled via primer walking. Chromatograms were scored by the ABI base caller with the sensitivity set at Q20. Any bases scored as "N" were visually read, and a base letter was assigned. If the chromatogram was unclear, the sequencing was repeated.

Complementary sequencing using pyrosequencing. To validate the Sanger sequencing results, we repeated the sequencing of a subset of isolates using pyrosequencing (PSQ), according to the standard manufacturer procedures adapted for the sequencing of XDR-TB-associated mutations (19, 20). Briefly, we used the PyroMark Q96 ID system (Qiagen, Valencia, CA) to perform PSQ of five of the eight Sanger sequencing gene targets identified in Table 1 (*katG*, *inhA* promoter, *rpoB*, *gyrA*, and *rrs*). *gyrB*, *tlyA*, and *eis* promoter were not evaluated with PSQ. Variant calling in PSQ was performed automatically with the IdentiFire software (Qiagen, Valencia, CA) according to the manufacturer's specifications.

Sanger sequence variant calling. Sanger sequence variant calling was performed by aligning the Sanger sequences against the *M. tuberculosis* strain H37Rv reference genome (GenBank accession no. NC_000962, NCBI). Variants included single nucleotide polymorphisms (SNPs) as well as insertions and deletions (indels). Synonymy analysis was performed for all variants falling in a protein-coding region of the genes *katG*, *rpoB*, *gyrA*, *gyrB*, and *tlyA*.

Sequence discordance resolution. The results from regions that were sequenced with both Sanger and pyrosequencing were compared. For every mutation identified by either Sanger sequencing or pyrosequencing, the matching sequence was examined in the other platform. Specific mutations found to be dissimilar (an SNP or indel in one and not the other, or different SNPs/indels) in each matching pair were considered discordant and excluded from the final analysis if they could not be resolved with repeated Sanger sequencing.

Mutation naming. Mutations were identified by their location relative to the reference *M. tuberculosis* H37Rv genome and the nucleotide change that was observed. The locations of SNPs and indels were described using the nucleotide coordinate system for H37Rv as well as the coordinate systems established in the literature for each of the individual genes (14–16).

Sensitivity and specificity of mutations. Isolates with the same phenotypic drug resistances and the same mutations were collated to examine associations across and between the clinical collection sites. The sensitivity and specificity values were determined by comparisons of the genotypic and phenotypic data. The confidence intervals for sensitivity and specificity were calculated using a score/efficient-score method with continuity correction (21, 22).

Human research conduct. This study was approved by the institutional review board of the University of California San Diego (UCSD).

TABLE 1 Genes associated with drug resistance phenotypes in XDR-TB strains and PCR primers used for Sanger sequencing[a]

Drug resistance phenotype(s)[b]	Primary gene and/or promoter associated with resistance in the literature[c]	PCR primer used to amplify gene/fragment for sequencing (5′ to 3′)	Gene/fragment size, including primers (bp)
INH^r	*katG*	CATGAACGACGTCGAAACAG CTCTTCGTCAGCTCCCACTC	270
INH^r	*inhA* promoter	AGAAAGGGATCCGTCATGGT GTCACATTCGACGCCAAAC	340
RIF^r	*rpoB*	CGTGGAGGCGATCACACCGCAGTT AGTGCGACGGGTGCACGTCGCGGACCT	215
MOX^r/OFX^r	*gyrA*	GGTGCTCTATGAAATGTTCG GCTTCGGTGTACCTCATCG	234
MOX^r/OFX^r	*gyrB*	CGATGTTCCAGGCGATACTT ATCTTGTGGTAGCGCAGCTT	163
AMK^r KAN^r CAP^r	*rrs*	GTAATCGCAGATCAGCAACG TTTTCGTGGTGCTCCTTAGAA	216
AMK^r KAN^r CAP^r	*eis* promoter/*eis*	AAATTCGTCGCTGATTCTCG CGCGACGAAACTGAGACC	387
AMK^r KAN^r CAP^r	*tlyA*	GTCTCTGGCCGAACTCGAAG ATTGTCGCCCAATACTTTTTCTAC	1,000

[a] XDR-TB, extensively drug-resistant tuberculosis.
[b] INH, isoniazid; RIF, rifampin; MOX, moxifloxacin; OFX, ofloxacin; AMK, amikacin; KAN, kanamycin; CAP, capreomycin.
[c] Data sources are references 14, 15, and 16.

RESULTS

DST. A total of 416 viable *M. tuberculosis* isolates were collected from India, Moldova, the Philippines, and South Africa and successfully underwent standardized liquid culture DST on the MGIT 960 platform. Table 2 indicates the number of viable *M. tuberculosis* isolates received from each site that were determined to be phenotypically resistant or susceptible to each of the study drugs. A total of 253/416 (61%) of the isolates were XDR TB, 98/416 (24%) were MDR TB only, 11/416 (3%) were monoresistant to either INH or RIF, and 36 were pansusceptible.

Sanger sequencing. DNA from all viable *M. tuberculosis* isolates was submitted for Sanger sequencing of eight gene regions (Table 1). We obtained evaluable sequences from an average of 408 isolates for each gene region (range, 336 to 430), totaling >850,000 bp of sequence. Detailed findings by country and gene are supplied in Tables S1 to S4 in the supplemental material. Only isolates with evaluable sequences and DST data were included in the analysis.

Pyrosequencing and discordance resolution. A total of 204 study isolates were selected at random for repeat sequencing with PSQ, allowing us to compare ~30,000 bp of sequence in six gene regions between the two platforms and identify 1,202 comparable sequence events (SNPs, indels, and wild-type DNA segments). Of these 1,202 matching events, 96% were identical. Sixty-one showed dissimilar SNPs, of which six were resolved by repeating Sanger sequencing, leaving a total of 55 discordant SNPs between Sanger and PSQ (4.6% of 1,202 comparable sequence events). These specific SNPs were assumed to be sequencing errors by one of the platforms and were excluded from further analysis, as they could not be resolved. We did not attempt to resolve the discordance between phenotypic DST and known resistance-conferring mutations by repeating the DST, but we did repeat the DST if the initial DST result was inconclusive as determined by standard MGIT 960 protocols.

Sensitivities and specificities of SNPs as markers of phenotypic drug resistance. The remaining SNPs identified in eight genes in 416 isolates were collated across sites, stratified by SNP type, and associated with the phenotypic resistance phenotypes in Tables 3 to 8. Detailed SNP data not included in these tables,

TABLE 2 Phenotypic drug susceptibility testing results for highly drug-resistant *M. tuberculosis* isolates collected from four multinational sites[a]

	Drug resistance (critical concn [mg/liter]) to the indicated antibiotic[b]													
	INH (0.1)		RIF (1.0)		MOX (0.25)		OFX (2.0)		AMK (1.0)		CAP (2.5)		KAN (2.5)	
	No. of isolates		No. of isolates		No. of isolates		No. of isolates		No. of isolates		No. of isolates		No. of isolates	
Study site	R	S	R	S	R	S	R	S	R	S	R	S	R	S
India	104	7	103	8	98	13	100	11	94	17	88	23	99	12
Moldova	83	8	80	11	68	23	67	23	31	60	32	59	76	15
Philippines	88	21	78	33	34	75	35	75	13	98	9	91	16	95
South Africa	96	7	95	8	92	11	90	13	92	11	90	13	95	8
Total	371	43	356	60	292	122	292	122	230	186	219	186	286	130

[a] Isolates were screened for phenotypic resistance (R, resistant; S, susceptible) on an MGIT 960 platform using WHO-recommended critical concentrations to define resistance.
[b] INH, isoniazid; RIF, rifampin; MOX, moxifloxacin; OFX, ofloxacin; AMK, amikacin; KAN, kanamycin; CAP, capreomycin.

TABLE 3 Sensitivities and specificities of SNPs for predicting phenotypic resistance to isoniazid in *M. tuberculosis* isolates from India, Moldova, the Philippines, and South Africa[a]

Observed mutation(s) by gene location	No. of INH^r isolates with mutation (n = 348)[b]	No. of INH^s isolates with mutation (n = 37)	Estimated sensitivity (% [95% CI])[c]	Estimated specificity (% [95% CI])
katG only				
315AGC→ACC	153	0	44 (38.7–49.4)	100 (88.3–100)
315AGC→ACA	2	0	0.6 (0.1–2.3)	100 (88.6–100)
inhA only				
−15C→T	31	1	8.9 (6.2–12.6)	97.4 (84.6–99.9)
−17G→T	2	0	0.6 (0.1–2.3)	100 (88.6–100)
katG and *inhA*				
315AGC→ACC/−15C→T	86	0	24.8 (20.4–29.7)	100 (88.6–100)
315AGC→ACC/−8T→A	38	0	11 (8–14.8)	100 (88.6–100)
315AGC→ACC/−17G→T	9	0	2.6 (1.3–5)	100 (88.6–100)
315AGC→ACC/−8T→C	5	0	1.4 (0.5–3.5)	100 (88.6–100)
315AGC→ACC/−34C→T	2	0	0.6 (0.1–2.3)	100 (88.6–100)
315AGC→ACC/−8T→G	2	0	0.6 (0.1–2.3)	100 (88.6–100)
315AGC→AAC/−15C→T	1	0	0.3 (0–1.9)	100 (88.6–100)
315AGC→ACA/−8T→C	1	0	0.3 (0–1.9)	100 (88.6–100)
315AGC→ACC/−34C→G	1	0	0.3 (0–1.9)	100 (88.6–100)
315AGC→ACC/−59G→C	1	0	0.3 (0–1.9)	100 (88.6–100)
Other *katG* and *inhA*	8	14	2.3 (1.1–4.7)	63.2 (46–77.7)
No mutations in *katG* or *inhA*	6	22		

[a] Phenotypic resistance determined by MGIT 960 drug susceptibility testing (DST) and WHO-recommended critical concentrations. SNPs, single nucleotide polymorphisms.
[b] INH, isoniazid.
[c] CI, confidence interval.

stratified by clinical site, are available in Tables S1 to S4 in the supplemental material.

INH resistance. A total of 238/347 (86%) of the phenotypic INH^r isolates contained the *katG* 315AGC→ACC SNP (Table 3). Only the *inhA* promoter SNPs −15C→T and −17G→T were independently associated with INH^r; all other *inhA* promoter mutations cooccurred with *katG* mutations. Four SNPs (*katG* 315AGC→ACC and AGC→ACA; *inhA* −15C→T, −17G→T) predicted phenotypic INH^r with 96% sensitivity. The individual mutations had specificity values of 97 to 100%.

TABLE 4 Sensitivities and specificities of SNPs for predicting phenotypic resistance to rifampin in *M. tuberculosis* isolates from India, Moldova, the Philippines, and South Africa[a]

Observed mutation(s) by gene location	No. of RIF^r isolates with mutation (n = 336)[b]	No. of RIF^s isolates with mutation (n = 55)	Estimated sensitivity (% [95% CI])	Estimated specificity (% [95% CI])
rpoB				
531TCG→TTG	231	1	68.8 (63.5–73.6)	98.2 (89–99.9)
516GAC→GGC/533CTG→CCG	27	0	8 (5.5–11.6)	100 (91.9–100)
516GAC→GTC	23	0	6.8 (4.5–10.2)	100 (91.9–100)
526CAC→TAC	18	0	5.4 (3.3–8.5)	100 (91.9–100)
531TCG→TGG	7	0	2.1 (0.9–4.4)	100 (91.9–100)
526CAC→CGC	6	0	1.8 (0.7–4)	100 (91.9–100)
513CAA→AAA	4	0	1.2 (0.4–3.2)	100 (91.9–100)
526CAC→GAC	3	0	0.9 (0.2–2.8)	100 (91.9–100)
522TCG→TTG	2	0	0.6 (0.1–2.4)	100 (91.9–100)
526CAC→TAC/526CAC→CGC	2	1	0.6 (0.1–2.4)	98.2 (89–99.9)
511CTG→CCG/512AGC→ACC/516GAC→TAC	1	0	0.3 (0–1.9)	100 (91.9–100)
513CAA→AAA/526CAC→GAC	1	0	0.3 (0–1.9)	100 (91.9–100)
526CAC→CTC	1	3	0.3 (0–1.9)	94.5 (83.9–98.6)
526CAC→TAC/509AGC→CGC	1	0	0.3 (0–1.9)	100 (91.9–100)
531TCG→TTG/515ATG→ATA	1	0	0.3 (0–1.9)	100 (91.9–100)
516GAC→TAC	0	4	0 (0–1.4)	92.7 (81.6–97.6)
526CAC→AAC/526CAC→CGC	0	1	0 (0–1.4)	98.2 (89–99.9)
526CAC→AAC	0	1	0 (0–1.4)	98.2 (89–99.9)
No mutations in *rpoB*	8	44		

[a] Phenotypic resistance determined by MGIT 960 drug susceptibility testing (DST) and WHO-recommended critical concentrations. SNPs, single nucleotide polymorphisms.
[b] RIF, rifampin.

TABLE 5 Sensitivities and specificities of primary SNPs predicting phenotypic resistance to moxifloxacin and/or ofloxacin in *M. tuberculosis* isolates from India, Moldova, the Philippines, and South Africa[a]

Observed mutation(s) by gene location	No. of FQ^r isolates w/ mutation (n = 284)[b]	No. of FQ^s isolates w/ mutation (n = 117)	Estimated sensitivity (% [95% CI])	Estimated specificity (% [95% CI])
gyrA				
94GAC→GGC[c]	91	0	32 (26.7–37.9)	100 (96–100)
90GCG→GTG[c]	82	1	28.9 (23.7–34.6)	99.1 (94.6–100)
94GAC→GCC[c]	25	0	8.8 (5.9–12.9)	100 (96–100)
94GAC→AAC[c]	23	0	8.1 (5.3–12.1)	100 (96–100)
94GAC→TAC[c]	11	0	3.9 (2–7)	100 (96–100)
91TCG→CCG[c]	6	0	2.1 (0.9–4.8)	100 (96–100)
94GAC→CAC[c]	5	0	1.8 (0.6–4.3)	100 (96–100)
88GGC→TGC[c]	3	0	1.1 (0.3–3.3)	100 (96–100)
90GCG→GTG	2	0	0.7 (0.1–2.8)	100 (96–100)
83AAC→.AC/94GAC→TAC[c,e]	1	0	0.4 (0–2.3)	100 (96–100)
94GAC→GCC	1	0	0.4 (0–2.3)	100 (96–100)
94GAC→GCC/90GCG→GTG[c]	1	0	0.4 (0–2.3)	100 (96–100)
94GAC→GGC	1	0	0.4 (0–2.3)	100 (96–100)
94GAC→GGC/112GGC→GGG/120GGC→GGG[c]	1	0	0.4 (0–2.3)	100 (96–100)
94GAC→GGC/125GCG→GCA[c]	1	0	0.4 (0–2.3)	100 (96–100)
94GAC→TAC	1	0	0.4 (0–2.3)	100 (96–100)
Other *gyrA* mutations	3	2	1.1 (0.3–3.3)	98.3 (93.3–99.7)
95AGC→ACC only[d]	26	107		
No mutations in *gyrA*	0	7		

[a] Phenotypic resistance determined by MGIT 960 drug susceptibility testing (DST) and WHO-recommended critical concentrations. *gyrB* SNPs with 100% specificity for phenotypic FQ resistance all cooccurred with *gyrA* SNPs and did not make any independent contributions to sensitivity; therefore, they were excluded here (see Tables S1 to S4 in the supplemental material for the *gyrB* SNPs observed in each country).
[b] FQ, fluoroquinolone.
[c] SNPs cooccurred with 95AGC→ACC SNP (excluded for brevity).
[d] SNP not associated with resistance to moxifloxacin or ofloxacin.
[e] "." denotes a deleted base.

RIF resistance. A total of 231/336 (69%) of RIF^r isolates contained the *rpoB* 531TCG→TTG SNP. Double mutations occurred in 32/336 (10%) of the RIF^r isolates (Table 4). Eleven unique SNPs had a combined sensitivity of 98% for predicting phenotypic RIF^r as defined by MGIT 960 DST. Three *rpoB* SNPs found in RIF^r isolates were also found in RIF^s isolates, which reduced their specificity to <100%. Two *rpoB* mutations (516GAC→TAC and 526CAC→AAC) occurred only in RIF^s isolates, as defined by MGIT 960 DST.

FQ resistance. Eight unique SNPs in *gyrA* codons 88 to 94 had a combined sensitivity of 90% for predicting phenotypic FQ^r, with only one SNP (*gyrA* 90GCG→GTG) having <100% specificity

TABLE 6 Sensitivities and specificities of SNPs for predicting phenotypic resistance to amikacin in *M. tuberculosis* isolates from India, Moldova, the Philippines, and South Africa[a]

Observed mutation(s) by gene location	No. of AMK^r isolates with mutation (n = 221)[b]	No. of AMK^s isolates with mutation (n = 172)	Estimated sensitivity (% [95% CI])	Estimated specificity (% [95% CI])
rrs only				
1401A→G	188	0	85.1 (79.5–89.4)	100 (97.3–100)
1484G→T	4	0	1.8 (0.6–4.9)	100 (97.3–100)
1401A→G/1462A→T/1486A→T	1	0	0.5 (0–2.9)	100 (97.3–100)
eis only				
−14C→T	10	1	4.5 (2.3–8.4)	99.4 (96.3–100)
−10G→A	2	3	0.9 (0.2–3.6)	98.3 (94.6–99.5)
−12C→T	1	38	0.5 (0–2.9)	77.9 (70.8–83.7)
rrs and *eis*				
1401A→G/−12C→T	2	0	0.9 (0.2–3.6)	100 (97.3–100)
1401A→G/−43A→T	1	0	0.5 (0–2.9)	100 (97.3–100)
Other *rrs*/*eis* mutations	0	5	0 (0–2.1)	97.1 (93–98.9)
No *rrs* or *eis* mutations	12	125		

[a] Phenotypic resistance determined by MGIT 960 drug susceptibility testing (DST) and WHO-recommended critical concentrations. SNPs, single nucleotide polymorphisms.
[b] AMK, amikacin.

TABLE 7 Sensitivities and specificities of SNPs for predicting phenotypic resistance to kanamycin in *M. tuberculosis* isolates from India, Moldova, the Philippines, and South Africa[a]

Observed mutation(s) by gene location	No. of KAN^r isolates with mutation (n = 276)[b]	No. of KAN^s isolates with mutation (n = 117)	Estimated sensitivity (% [95% CI])	Estimated specificity (% [95% CI])
rrs only				
1401A→G	188	0	68.1 (62.2–73.5)	100 (96–100)
1484G→T	4	0	1.4 (0.5–3.9)	100 (96–100)
1401A→G/1462A→T/1486A→T	1	0	0.4 (0–2.3)	100 (96–100)
eis only				
−12C→T	38	1	13.8 (10–18.5)	99.1 (94.6–100)
−14C→T	11	0	4 (2.1–7.2)	100 (96–100)
−10G→A	5	0	1.8 (0.7–4.4)	100 (96–100)
rrs and *eis*				
1401A→G/−12C→T	2	0	0.7 (0.1–2.9)	100 (96–100)
1401A→G/−43A→T	1	0	0.4 (0–2.3)	100 (96–100)
Other *rrs*/*eis* mutations	2	3	0.7 (0.1–2.9)	97.4 (92.1–99.3)
No *rrs* or *eis*	24	113		

[a] Phenotypic resistance determined by MGIT 960 drug susceptibility testing (DST) and WHO-recommended critical concentrations. SNPs, single nucleotide polymorphisms.
[b] KAN, kanamycin.

(Table 5). Twenty-six FQ^r isolates had no SNPs in the region of *gyrA* examined, other than the codon 95ACC→AGC, which is known to be a lineage-specific marker not related to resistance (16). We observed several SNPs in *gyrB* (see Tables S1 to S4 in the supplemental material) that were associated with FQ^r with 100% specificity, but as they all cooccurred with *gyrA* SNPs (e.g., *gyrA* 91TCG→CCG, 95AGC→ACC/*gyrB* 543GCG→GTG; see Table S1 in the supplemental material), they did not make independent contributions to sensitivity and were therefore excluded from Table 5.

AMK, KAN, and CAP resistance. While there was substantial phenotypic cross-resistance between the injectable drugs AMK, KAN, and CAP, we reported SNPs related to resistance to each of these drugs separately, as there are important distinctions in the SNPs associated with resistance to these individual drugs (Tables 6 to 8). The *rrs* SNPs 1401A→C and 1484G→T predicted amikacin and CAP^r with 89% and 90% sensitivities, respectively, but only 71% of the observed phenotypic KAN^r was explained by these *rrs* SNPs. Adding the *eis* −14C→T SNP gave a combined sensitivity of 93% for predicting AMK^r without significantly decreasing specificity (Table 6), and adding the *eis* promoter SNPs −10G→A, −12C→T, and −14C→T increased the sensitivity for KAN^r detection to 91%. The *eis* −10G→A and −12C→T SNPs had almost 100% specificity for KAN^r (Table 7) but were not specific for AMK^r or CAP^r, as they occurred more often in AMK^s and CAP^s isolates than in AMK^r and CAP^r isolates. Similar to previous studies (23), SNPs observed in the *tlyA* gene in isolates resistant to AMK, KAN, and/or CAP were not found to be sensitive or specific for phenotypic resistance in this analysis (see Tables S1 to S4 in the supplemental material).

Regional variation in SNP frequencies. The sensitivities of in-

TABLE 8 Sensitivities and specificities of SNPs for predicting phenotypic resistance to capreomycin in *M. tuberculosis* isolates from India, Moldova, the Philippines, and South Africa[a]

Observed mutation(s) by gene location	No. of CAP^r isolates with mutation (n = 210)[b]	No. of CAP^s isolates with mutation (n = 183)	Estimated sensitivity (% [95% CI])	Estimated specificity (% [95% CI])
rrs only				
1401A→G	181	7	86.2 (80.6–90.4)	96.2 (92–98.3)
1484G→T	4	0	1.9 (0.6–5.1)	100 (97.4–100)
1401A→G/1462A→T/1486A→T	1	0	0.5 (0–3)	100 (97.4–100)
eis only				
−12C→T	6	33	2.9 (1.2–6.4)	82 (75.5–87.1)
−14C→T	1	10	0.5 (0–3)	94.5 (89.9–97.2)
−10G→A	1	4	0.5 (0–3)	97.8 (94.1–99.3)
rrs and *eis*				
1401A→G/−12C→T	2	0	1 (0.2–3.8)	100 (97.4–100)
1401A→G/−43A→T	1	0	0.5 (0–3)	100 (97.4–100)
Other *rrs*/*eis* mutations	0	5	0 (0–2.2)	97.3 (93.4–99.0)
No *rrs* or *eis* mutations	13	124		

[a] Phenotypic resistance determined by MGIT 960 drug susceptibility testing (DST) and WHO-recommended critical concentrations. SNPs, single nucleotide polymorphisms.
[b] CAP, capreomycin.

dividual SNPs associated with resistance varied only moderately between clinical sites, with a few notable exceptions (see Tables S1 to S4 in the supplemental material). However, combinations of SNPs still had the same overall sensitivities within and across sites. The most notable exception to this finding was the sensitivities of the *rrs* and *eis* promoter mutations as predictors of KAN^r at the four sites. In India and South Africa, KAN^r was predicted largely by *rrs* mutations (86 to 89%), with *eis* contributing only 3 to 4%, while in Moldova, *eis* SNPs had 62% sensitivity and *rrs* contributed only 28%. In the Philippines, no *eis* SNPs were found in KAN^r isolates (see Tables S1 to S4 in the supplemental material).

DISCUSSION

The performance of rapid molecular diagnostics based on the detection of mutations associated with phenotypic resistance depends on the frequency of those mutations in the pathogen population (sensitivity) and the strength of the association between the mutations and phenotypic resistance (specificity). Our results suggest that molecular diagnostic platforms with the capability to detect a minimum of 15 SNPs in *katG*, *inhA* promoter, and *rpoB* should detect INH and RIF resistance with 96 to 98% sensitivity and approaching 100% specificity, while the detection of an additional 13 SNPs in *gyrA*, *rrs*, and *eis* promoter should identify approximately 90% of FQ, AMK, KAN, and CAP resistance with almost 100% specificity. Our multinational study, while not exhaustive, includes clinical M/XDR-TB isolates from local and national archives in four regions of the world with some of the highest M/XDR-TB burdens. These results should provide a solid foundation for performance expectations for existing molecular diagnostic platforms globally and serve as a guide in the development of further studies on mutations associated with phenotypic resistance, clinical correlation studies, and new rapid diagnostic technologies. Specific exceptions and cautions are noted below.

INH resistance. The combined sensitivity of *katG* and *inhA* promoter mutations for the detection of the phenotypic INH^r that we observed (96%) is consistent with the cumulative frequency of these mutations in the published literature (24–26). There are, however, some recent studies on international isolates which suggest that using only *katG* and *inhA* promoter SNPs will yield sensitivities of <90% due to isolates that do not have these SNPs (27). The *katG* and *inhA* promoter SNPs we examined are all detected by the currently available line probe assays, such as the Hain Lifescience assay, which is commercially available and WHO approved for INH^r detection (9). Based on the observed sensitivity and specificity of these SNPs in our study, we would expect that LPAs, such as the Hain Lifescience MTBDR*plus*, would have a sensitivity of around 90% and not the 85% (95% confidence interval [CI], 77% to 90%) sensitivity that was observed in the WHO review of pooled studies (9). The reason for this discrepancy might be a different global frequency of *katG/inhA* mutations than we observed, but it might also have to do with the ability of LPAs to detect these mutations with 100% fidelity when they are present. GCDD completed a large clinical study of the Hain Lifescience LPAs (>1,000 patients) in July 2013 that includes a comprehensive sequencing evaluation that should shed some light on this issue in the near future.

RIF resistance. While most of the *rpoB* SNPs we observed were only found in phenotypically resistant isolates, two *rpoB* SNPs (516GAC→TAC and 526CAC→AAC) only occurred in isolates defined as RIF susceptible by MGIT 960 DST and therefore appear to be poor predictors of phenotypic resistance. However, the interpretation of these data is complicated by the complex phenotypes that have been observed in isolates with these mutations. It has been well documented that most SNPs in the 81-bp resistance-determining region (RDR) of the *rpoB* gene confer phenotypic RIF^r (28, 29). Thus, molecular diagnostics that detect *rpoB* mutations, such as the LPAs and the GeneXpert assay, have demonstrated high sensitivities and specificities as tools for predicting phenotypic RIF^r in clinical samples (9, 30). It is important to note, however, that not all *rpoB* SNPs confer the same level of resistance. Certain *rpoB* mutations, such as the 516GAC→TAC and 526CAC→AAC mutations that we observed, appear to confer low-level resistance, as measured by the low MICs of the isolates (29). These MICs are usually just below or distributed across the WHO critical concentration for liquid DST, which means these isolates can go undetected by MGIT 960 DST and appear to be susceptible (31–33). The clinical relevance of these mutations still needs to be determined, however. Recent retrospective analyses of treatment outcomes in patients with isolates containing low-MIC mutations, such as the 516GAC→TAC and 526CAC→AAC mutations, appear to suggest that these mutations might still be associated with poor treatment outcomes in standard RIF-based first-line treatment regimens (32, 34, 35), but these studies had many unmeasured confounding factors that might have affected the results. In the long term, it will be critical to address the discordant phenotypic standards by which isolates with low-level MIC resistance are evaluated and to conduct studies on the clinical relevance of these mutations. In the short term, *M. tuberculosis* isolates with these mutations should probably be considered low-level resistant and likely treatable with optimized RIF dosing based on the RIF MIC of each isolate (36). Molecular diagnostics with the ability to distinguish between *rpoB* SNPs with high fidelity, coupled with clinical interpretation algorithms based on established SNP-MIC relationships, will be valuable clinical tools.

In contrast to the established low-MIC SNPs described above, the *rpoB* 531TCG→TTG and 526CAC→TAC/526CAC→CGC SNPs that we observed are documented to be high-MIC mutations in both solid and liquid media (29, 31, 33). The single instances of these mutations we observed occurring in two isolates designated susceptible by MGIT 960 DST (Table 4) were possibly the result of DST errors. This highlights a complication of this kind of analysis using phenotypic DST as the gold standard, when it is known that phenotypic DST has sensitivity and specificity limitations as well (37).

FQ resistance. Eight unique SNPs in *gyrA* codons 88 to 94 had a combined sensitivity of 90% for predicting phenotypic FQ^r with 99 to 100% specificity in our multinational sample of isolates. Only one of these SNPs (90GCG→GTG) had <100% specificity (99%), as it was observed in 82 resistant isolates but also in a single phenotypically susceptible isolate as determined by MGIT 960 DST. As this particular isolate had to have its DST repeated to get a conclusive result, it is possible this is a DST error, possibly resulting from undetected heteroresistance.

Molecular diagnostic methods with the ability to detect these eight SNPs should prove to be very sensitive and specific clinical tools for the rapid diagnosis of phenotypic resistance. Approximately 10% of the FQ^r isolates we evaluated did not have mutations in the regions of *gyrA* and *gyrB* we examined, which based on a recent review of mutations associated with FQ^r (16) suggests it is unlikely that molecular diagnostics detecting only *gyrA*/*gyrB* SNPs can yield >90% sensitivity. However, it is important to under-

stand this limitation in the context of the currently available alternatives for diagnosing FQ^r. All available alternatives are based on phenotypic DST, which is costly, slow, and out of reach for many national TB programs that are placing MDR-TB patients on empirical treatment with a FQ without knowing if that treatment choice will be effective. While it is clear that we need to look for mutations outside the QRDR region of *gyrA* to improve the sensitivity of molecular diagnostics for FQ^r detection, we also need to use the tools we currently have at our disposal (e.g., Hain Lifescience MTBDR*sl*, PSQ) for the rapid and specific detection of phenotypic resistance in the majority of the patients with FQ^r.

AMK, KAN, and CAP resistance. Based on our findings, detection of the *rrs* SNPs 1401A→C and 1484G→T should effectively identify 89 to 90% of the phenotypic resistance to AMK and CAP in TB patients with resistance to these drugs. In our study, these mutations predicted phenotypic AMK^r with 100% specificity, but these mutations were also present in 7/183 (4%) of "CAP-susceptible" isolates (as determined by MGIT 960 DST), suggesting <100% specificity for the prediction of CAP-resistant phenotypes. One possible explanation for the observed lack of specificity for predicting phenotypic CAP resistance may lie in the WHO-recommended critical concentration for MGIT 960 DST that we used for our study. Since the current critical concentration was accepted by the WHO, it has been demonstrated that this critical concentration is substantially higher than the epidemiological cutoff (ECOFF) that separates wild-type *M. tuberculosis* from those with mutations conferring CAP resistance, which might result in non-wild-type isolates being classified as CAP^s (38, 39). Interpreted in the context of our findings, it is possible that the WHO-recommended critical concentration we used misclassified CAP-resistant organisms as susceptible, resulting in a reduced specificity of the mutation for predicting phenotypic resistance. More needs to be understood about the relationships of these mutations and their CAP-specific MICs to improve the clinically relevant predictive value of molecular diagnostics for detecting phenotypic CAP^r.

Like the INH^r that is caused by both *katG* and *inhA* promoter mutations, phenotypic KAN^r is caused by mutations in both *rrs* and the *eis* promoter (40, 41). Due to regional variations in the frequencies of *eis* and *rrs* mutations in the KAN^r isolates that we observed, we predict that rapid diagnostic methods, such as the Hain Lifescience MTBDR*sl*, which detect only *rrs* SNPs and not *eis* promoter SNPs, will underestimate KAN^r significantly in countries like Moldova (which had mostly *eis* mutations in KAN^r isolates). However, they should still have a sensitivity of ~90% for KAN^r in countries, such as India and South Africa, where we observed KAN^r being associated mostly with *rrs* mutations. The lack of *eis* probes in these LPAs likely contributed to the poor sensitivity (~50%) recently reported for the MTBDR*sl* assay for the detection of aminoglycoside-resistant isolates from the country of Georgia (42). Adding *eis* promoter SNPs (−10G→A, −12C→T, and −14C→T) to existing second-line LPAs and future molecular diagnostics would increase their utility globally and provide added confidence for the detection of KAN^r in regions where *rrs*/*eis* frequencies are not yet known.

Conclusion. Based on our findings, it is clear that molecular diagnostics based on the detection of <30 SNPs in 6 genes should be able to predict clinically relevant phenotypic resistance to INH, RIF, FQ, AMK, KAN, and CAP with 90 to 98% sensitivity and almost 100% specificity in most regions. The existing Hain Lifescience LPAs (MTBDR*plus* and MTBDR*sl*) detect most of these mutations, with some limitations noted above, and new sequencing-based platforms, such as PSQ, have the ability to detect and discriminate all of these SNPs (19, 20, 43). Given that <25% of incident MDR-TB cases are currently being diagnosed based on phenotypic DST alone, it seems that there is a clear clinical role for molecular diagnostics, at least in early treatment decisions while waiting for phenotypic DST results. Since empirical studies have clearly demonstrated that early treatment with appropriate therapy can halt TB transmission almost immediately, while undetected, inappropriately treated DR TB continues to be transmitted (5, 44), adopting imperfect but effective rapid diagnostic methods may help reduce transmission and is in the best interest of the public.

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