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Molecular Detection of Mutations Associated with First- and Second-Line Drug Resistance Compared with Conventional Drug Susceptibility Testing of *Mycobacterium tuberculosis*▽†§

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The emergence of multi- and extensively drug-resistant tuberculosis is a significant impediment to the control of this disease because treatment becomes more complex and costly. Reliable and timely drug susceptibility testing is critical to ensure that patients receive effective treatment and become noninfectious. Molecular methods can provide accurate and rapid drug susceptibility results. We used DNA sequencing to detect resistance to the first-line antituberculosis drugs isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) and the second-line drugs amikacin (AMK), capreomycin (CAP), kanamycin (KAN), ciprofloxacin (CIP), and ofloxacin (OFX). Nine loci were sequenced: *rpoB* (for resistance to RIF), *katG* and *inhA* (INH), *pncA* (PZA), *embB* (EMB), *gyrA* (CIP and OFX), and *rrs*, *eis*, and *tlyA* (KAN, AMK, and CAP). A total of 314 clinical *Mycobacterium tuberculosis* complex isolates representing a variety of antibiotic resistance patterns, genotypes, and geographical origins were analyzed. The molecular data were compared to the phenotypic data and the accuracy values were calculated. Sensitivity and specificity values for the first-line drug loci were 97.1% and 93.6% for *rpoB*, 85.4% and 100% for *katG*, 16.5% and 100% for *inhA*, 90.6% and 100% for *katG* and *inhA* together, 84.6% and 85.8% for *pncA*, and 78.6% and 93.1% for *embB*. The values for the second-line drugs were also calculated. The size and scope of this study, in numbers of loci and isolates examined, and the phenotypic diversity of those isolates support the use of DNA sequencing to detect drug resistance in the *M. tuberculosis* complex. Further, the results can be used to design diagnostic tests utilizing other mutation detection technologies.

Two critical components of tuberculosis (TB) control are prompt identification of new cases and rapid implementation of effective treatment regimens to interrupt transmission. The standard treatment regimen combines four first-line antibiotics, isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB), and renders patients with tuberculosis noncontagious when they are properly administered. Inadequate treatment can select for drug-resistant bacilli (acquired resistance), and those resistant organisms can be transmitted to other individuals (primary resistance). Although drug resistance is disconcerting, the emergence of multidrug-resistant (MDR) TB, defined as resistance to both INH and RIF, is especially worrisome, as it leads to more complex and costly treatment regimens. In 2008, the World Health Organization (WHO) estimated that there were 11.1 million cases of tuberculosis worldwide, of which 440,000 cases were MDR TB (43). MDR TB complicates treatment because ineffective first-line antibiotics must be replaced with second-line drugs that are more costly and that can lead to more adverse side effects. These drugs include several fluoroquinolone (FQ) compounds as well as the injectable drugs amikacin (AMK), capreomycin (CAP), and kanamycin (KAN). In 2006, the Centers for Disease Control and Prevention (CDC) and WHO defined extensively drug-resistant (XDR) TB as MDR TB that is also resistant to any FQ and one of the second-line injectable drugs (6, 41). The full extent of XDR TB is unknown because many countries lack sufficient laboratory system capacity, especially for diagnosis. The available data show that 5.4% of MDR TB cases fit the XDR TB definition (43), with 55 countries or territories reporting at least one case by the end of 2008 (42).

The control of drug-resistant TB, in any form, requires accurate and prompt diagnosis of the type of resistance. The timeliness of culture-based drug susceptibility testing (DST) is constrained by the slow growth characteristic of *Mycobacterium tuberculosis*, which can take 2 to 4 weeks, depending on the method of testing. DST of certain drugs can also be technically challenging and, in resource-limited areas, cost prohibitive. The use of molecular methods to identify mutations associated with drug resistance can decrease diagnostic delay and, in some cases, may prove to be more specific than phenotypic DST. The great potential of genetic testing to rapidly diagnose drug resistance has been the impetus behind a tremendous amount of basic and applied research. The result of this area of research has been the development of numerous commercial and

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laboratory-designed diagnostic assays. These assays are increasingly being used in clinical and reference laboratories.

In September 2009, our laboratory at CDC began offering a new, Clinical Laboratory Improvement Amendments (CLIA)-compliant reference service: DNA sequencing of drug resistance-associated loci (8). Prior to implementation, to verify this molecular detection of drug resistance (MDDR) service, we sequenced all or part of nine loci in 314 *M. tuberculosis* complex isolates. The loci were chosen on the basis of their demonstrated association with drug resistance and included *rpoB* (RIF), *katG* and *inhA* (INH), *pncA* (PZA), *embB* (EMB), *gyrA* (FQ), and *rrs*, *eis*, and *tlyA* (second-line injectables). Previous studies comparing phenotypic DST results to molecular data have been fragmented in scope, being focused on a limited array of loci in a comparatively small number of primarily or exclusively drug-resistant isolates. In the study described in this report, we analyzed a large set of clinical isolates representing a variety of antibiotic resistance patterns, including pansusceptible, poly-drug-resistant (isolates that were resistant to two or more drugs but that did not meet the definition of MDR TB), MDR, and XDR isolates. The phenotypic and genotypic results were compared to determine the specificity and sensitivity for each locus studied.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. tuberculosis* H37Rv, 60 WHO proficiency test isolates from 2007 and 2008 (representing 30 unique patient isolates in duplicate), and 254 stored clinical isolates (collected from 2000 to 2008) were selected from the culture collection at the Mycobacteriology Laboratory Branch, Division of Tuberculosis Elimination, CDC. Samples were chosen to represent a variety of antibiotic resistance patterns from both U.S. and non-U.S. laboratories. The 314 isolates were grown in 5 ml of Middlebrook 7H9 broth (7H9) supplemented with 10% (vol/vol) albumin-dextrose-catalase enrichment (Difco Laboratories) and 0.05% (vol/vol) Tween 80 (Sigma-Aldrich) at 37°C until they reached an approximate optical density at 600 nm of 1.0 (corresponding to 5×10^8 CFU/ml).

Drug susceptibility testing. Antituberculosis DST was completed according to the Clinical and Laboratory Standards Institute (CLSI) standard (24). Testing of all clinical isolates was completed at the time of their submittal to the CDC. Susceptibility was determined via the indirect agar proportion method utilizing Middlebrook 7H10 agar supplemented individually with the following drugs: RIF (1 μg/ml), INH (0.2, 1, and 5 μg/ml), EMB (5 μg/ml), ofloxacin (OFX; 2 μg/ml), ciprofloxacin (CIP; 2 μg/ml), KAN (5 μg/ml), CAP (10 μg/ml), and AMK (4 μg/ml). PZA was tested using the Bactec 460 (100 μg/ml), MGIT (100 μg/ml), or agar proportion (25 μg/ml) method, on the basis of testing practices at the time of isolate receipt.

DNA isolation, spoligotyping, amplification, and sequencing of loci. DNA was isolated from 7H9 subcultures by mechanical cell disruption, as previously described (23). In short, 1 ml of each subculture was transferred to a 2-ml Lysing Matrix B tube (MP Biomedical, Inc.), heat killed, and mixed with 400 μl Tris-EDTA buffer and 400 μl chloroform. The mixture was thoroughly agitated using a FastPrep 120 apparatus (MP Biomedicals) for 20 s at a setting of 4.0. The samples were centrifuged at 8,000 × *g* for 5 min, and the DNA-containing aqueous layer was collected and stored at 4°C. Families of study isolates were determined by spoligotyping as previously described (7).

The following nine loci were amplified by PCR: *rpoB* (RIF), *katG* and *inhA* (INH), *embB* (EMB), *gyrA* (FQ), *rrs* (KAN, CAP, and AMK), *eis* (KAN), *tlyA* (CAP), and *pncA* (PZA). Of the nine loci, only the drug resistance-determining regions of *rpoB* (rifampin resistance-determining region [RRDR]), *embB* (ethambutol resistance-determining region [ERDR]), and *gyrA* (quinolone resistance-determining region [QRDR]), the promoters of *inhA* and *eis*, and regions with established resistance-associated mutations of *katG* and *rrs* were amplified using locus-specific primers (see Table S1 in the supplemental material). The complete open reading frames of *tlyA* and *pncA* were amplified due to the established presence of resistance-associated mutations throughout the loci (see Table S1 in the supplemental material). Each 25-μl PCR mixture contained 12.5 μl HotStarTaq master mix (Qiagen), 1.5 μl of the forward and reverse 5 μM primers, 8.5 μl distilled H_2O (dH_2O), and 1 μl of genomic DNA. The amplification parameters included an initial denaturation step at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s, with a final elongation step at 72°C for 7 min. All primers were synthesized by the Biotechnology Core Facility at CDC (see Table S1 in the supplemental material). PCR products were analyzed on 1.5% agarose-Tris-EDTA gels and stained with ethidium bromide. Verified PCR products were treated with ExoSAP-IT reagent (USB Corporation), using the manufacturer's protocol, to remove unincorporated primers and nucleotides. The treated products were then diluted 1:10 with dH_2O and used as the template for DNA sequencing.

Sequencing reactions (20-μl reaction mixtures) were completed with an ABI BigDye Terminator (version 3.1) cycle sequencing kit, and the reaction mixtures included 4 μl of BigDye Terminator (version 3.1) reagent (ABI), 2 μl of 1.6 μM primer (with the same sequences as the primers used for PCR), 2 μl of 5× reaction buffer (ABI), 11 μl of dH_2O, and 1 μl of PCR template. PCR products were sequenced with a forward primer and a reverse primer at each locus for maximum coverage and reproducibility of results. The reactions were cycled according to manufacturer guidelines. The unincorporated terminators were removed from the completed reactions by treatment with a BigDye Xterminator kit (ABI), using the manufacturer's protocol, and were examined using an ABI 3130xl genetic analyzer with standard run conditions for electrophoresis and data collection.

Analysis. Sequence data produced by the ABI 3130xl genetic analyzer were reviewed for confidence levels with an ABI sequence scanner, and chromatograms were analyzed for the presence or absence of mutations by comparison with published sequences of H37Rv using the SeqMan alignment application of the DNAStar Lasergene (version 8.0) program. Genotypic data for each isolate at a particular locus were recorded in a Microsoft Office Excel 2003 spreadsheet. Mixed peaks within a chromatogram were treated as being true if they were reproducible in both the forward and reverse directions. The genetic data, spoligotype results, and phenotypic data sets were then compiled in separate tables within a Microsoft Office Access 2003 database and analyzed further with the analysis function of Epi Info (version 3.5.1, 2008) software. Repeat testing was performed on any isolate for which the genotypic result predicted resistance but it was determined to be susceptible by the initial DST. This was true for all drugs except PZA, due to variability in testing methods. Sensitivity and specificity values were determined by comparison of the genotypic and phenotypic data sets.

RESULTS

Drug susceptibility testing and DNA sequencing. We analyzed 314 clinical isolates, including 60 from the WHO molecular proficiency testing panels (2007 to 2008) and 254 from the isolates stored at CDC (2000 to 2008). The isolates were received from both U.S. (134 isolates; 43%) and non-U.S. (167; 53%) laboratories (geographic origin data were unavailable for 72 [23%] of the study isolates). Spoligotyping was performed on the entire study set and determined that the most highly represented families were the East Asian Beijing (127; 40.4%), EuroAmerican (54; 17.2%), and EuroAmerican (Latin American and Mediterranean; 44; 14.0%). The IndoOceanic (18; 5.7%), East African Indian (7; 2.2%), EuroAmerican S (2; 0.6%), IndoOceanic India (1; 0.3%), and IndoOceanic Manila (1; 0.3%) lineages were less represented (see Table S2 in the supplemental material). Spoligotyping also revealed one *M. bovis* isolate, 14 unknown spoligotype patterns, and three isolates for which no result could be obtained (see Table S2 in the supplemental material).

In addition to spoligotyping, phenotypic DST was completed on all study isolates for six of the nine study drugs: RIF, INH, CIP, KAN, CAP, and EMB. Testing was not completed on all isolates for OFX (69; 21.9%), as it was not fully incorporated into the CDC testing regimen until 2002; AMK (1; 0.3%), due to a lack of growth from one original isolate, and PZA (122; 39%), due to a lack of testing for the WHO panel samples, as

TABLE 1. Number of clinical *M. tuberculosis* isolates stratified by resistance to nine antibiotics, mutation, and validity values for each respective locus

Drug	Locus	No. of isolates				Accuracy values	
		Resistant		Susceptible			
		With mutation	Without mutation	With mutation	Without mutation	Sensitivity (%)	Specificity (%)
RIF	*rpoB*	169[a]	5	9	131	97.1	93.6
INH	*katG*	181	31	0	102	85.4	100
	inhA	35	177	0	102	16.5	100
	katG and/or *inhA*	192	20	0	102	90.6	100
EMB	*embB*	121[b]	33[c]	11[d]	149[e]	78.6	93.1
PZA[f]	*pncA*	55	10	15[g]	109[h]	84.6	85.8
CIP and OFX	*gyrA*	80	18	5	211	81.6	97.7
KAN	*rrs*	64	47	2	201	57.7	99.0
	eis	32	79	6	197	28.8	97.0
	rrs or *eis*	96	15	8	195	86.5	96.1
AMK	*rrs*	63	7	3	241	90.0	98.8
CAP	*rrs*	38	31	28	217	55.1	88.6
	tlyA	7	62	3	242	10.1	98.8
	rrs and/or *tlyA*	42[i]	27	31	214	60.9	87.3
MDR (RIF and INH)	*rpoB* and *katG* and/or *inhA*	148	15	NA[j]	NA	90.8	94.7

[a] Includes 15 isolates with two mutations.
[b] Excludes 3 isolates in which only the Glu378Ala polymorphism was identified.
[c] Includes 3 isolates with only the Glu378Ala polymorphism.
[d] Excludes 16 isolates in which only the Glu378Ala polymorphism was identified.
[e] Includes 16 isolates with only the Glu378Ala polymorphism.
[f] Includes 192 isolates with drug susceptibility results.
[g] Excludes 3 isolates with silent mutations.
[h] Includes 3 isolates with silent mutations.
[i] Includes 2 isolates with a mutation in each locus.
[j] NA, not applicable.

well as variation in CDC testing methods. Of the tested isolates, 55 (16%) were susceptible to all study antibiotics, 163 (52%) were found to be MDR *M. tuberculosis*, and 10 (3%) were determined to be XDR *M. tuberculosis*. The remaining 96 (29%) isolates included 19 (19.8%) monoresistant and 77 (80.2%) poly-drug-resistant isolates. DNA sequencing of the nine loci in the study was 90.8% sensitive and 94.7% specific for the detection of MDR *M. tuberculosis* isolates (Table 1) and was 40.0% sensitive and 99.3% specific for the detection of XDR *M. tuberculosis* isolates. DST and DNA sequencing agreement varied for each locus and drug combination. Whenever discordance between the two methods was detected, repeat testing was initiated for both techniques with all drugs except PZA. If the repeat test results were in conflict with the original data, a third round of testing was completed, with the final value representing two out of the three iterations. The only exceptions to this practice were the WHO panel isolates, which were not subjected to repeat drug susceptibility testing. Results for each of the nine drugs and their respective loci are presented individually in the succeeding sections.

Rifampin and *rpoB*. Since the majority of strains resistant to RIF harbor mutations within the RRDR of *rpoB*, this region and flanking sequence were analyzed in this study. A total of 174 (55.4%) of the isolates were RIF resistant and included 163 MDR and 10 XDR *M. tuberculosis* isolates. The vast majority (169/175; 97.1%) of RIF-resistant isolates harbored at least one mutation within the RRDR of *rpoB*, while 5 of the resistant isolates lacked such a mutation (see Table S3 in the supplemental material). A total of 26 nonsynonymous single nucleotide polymorphisms (nSNPs), one 3-bp insertion, and a silent mutation were identified among the study isolates. Fifteen of the resistant isolates harbored two missense mutations represented by 12 unique pairs and 3 of the same combination (Gln513His and Leu533Pro). In seven of the double mutants, one of the mutations occurred outside the RRDR, while the other mutation was located within that region. One isolate with a silent mutation (Phe514Phe) also had a Ser531Leu substitution. When all mutations, both single and double, are considered, a total of 31 *rpoB* genotypes were identified (see Table S3 in the supplemental material). The three most frequently observed mutations accounted for 75% of the RIF-resistant isolates in this study (Table 2). Among the 140 RIF-susceptible isolates, 9 were found to have a mutation within the *rpoB* gene, including 4 isolates with a His526Asn substitution, 2 each with either a His526Leu or Leu533Pro substitution, and 1 with a Leu511Pro substitution. Detection of nSNPs within the RRDR of *rpoB* exhibited a sensitivity and specificity of 97.1% and

TABLE 2. Most frequently identified mutations within nine *M. tuberculosis* drug resistance-associated loci among isolates resistant to that antibiotic for which each locus serves as a resistance marker

Drug	Locus	Mutation	Frequency (no. of isolates)	Relative frequency[a] (%)
RIF	*rpoB*	Ser531Leu	108	62.1
		His526Tyr	20	11.5
		Asp516Val	12	6.9
INH	*katG*	Ser315Thr	176	83.0
	inhA	C(−15)T	32	15.1
		T(−8)C	3	1.4
EMB	*embB*	Met306Val	55	42
		Met306Ile	27	20.6
PZA	*pncA*	Frameshifts	15	23.1
		Gln10Pro	4	6.2
CIP and OFX	*gyrA*	Asp94Gly	28	28.6
		Asp90Val	25	25.5
		Asp94Ala	10	10.2
KAN	*rrs*	A1401G	62	55.9
	eis	G(−10)A	14	12.6
		C(−14)T	11	9.9
AMK	*rrs*	A1401G	62	88.6
CAP	*rrs*	A1401G	34	49.3
	tlyA	Various	NA[b]	NA

[a] Compared with the total number of isolates resistant to drug of interest.
[b] NA, not applicable.

93.6%, respectively, for predicting phenotypic RIF results (Table 1).

Isoniazid and *katG* and *inhA*. INH is classified as a prodrug and is activated by the catalase-peroxidase encoded by *katG*. Mutations within *katG*, especially at codon 315, can result in loss of catalase activity and INH resistance. The target of activated INH is enoyl-acyl carrier protein reductase (InhA), and increased expression of *inhA* as a result of promoter mutations leads to resistance via a drug titration mechanism. We sequenced a region of *katG* encompassing codon 315 and the *inhA* promoter because it has been well established that most INH-resistant isolates possess mutations in one or both of those regions. A total of 212 isolates were resistant to at least 0.2 μg/ml INH, of which 192 (90.5%) contained a *katG* and/or an *inhA* mutation (see Table S4 in the supplemental material). Twenty-four (11.3%) of the resistant isolates harbored a mutation in both loci, while 157 (81.8%) had only *katG* mutations and 11 (5.7%) had only *inhA* mutations. The remaining 20 (9.4%) INH-resistant isolates were wild type in both loci. As anticipated, the most prevalent *katG* alteration was the substitution of threonine for serine at amino acid 315. This amino acid substitution resulted from a codon change from AGC to ACC in 165 (86%) isolates and to ACA in 9 (5%) isolates. Other *katG* codon 315 mutations observed were substitution with asparagine (AAC) in two isolates and isoleucine (ATC) in one isolate. Three isolates had an Ile335Val change in combination with a Ser315Thr substitution. One resistant isolate had a single *katG* missense mutation in a codon other than 315, resulting in the substitution of serine (AGT) for glycine (GGT) at codon 273. No *katG* mutations were found in the 102 INH-susceptible isolates. Detection of an nSNP within the region of *katG* analyzed exhibited a sensitivity of 85.4% and a specificity of 100% (Table 1).

Thirty-five of the INH-resistant isolates had a mutation within the *inhA* promoter, 32 (91.4%) of which were a cytosine-to-thymine transition at the nucleotide positioned 15 bases upstream of the start codon [C(−15)T] (see Table S4 in the supplemental material). The remaining three INH-resistant isolates had a transition from thymine to cytosine 8 nucleotide bases upstream from the start codon [T(−8)C]. Twenty-one of the isolates with a C(−15)T mutation also had a *katG* Ser315Thr substitution. All 21 of these isolates were resistant to 5 μg/ml isoniazid. None of the INH-susceptible isolates possessed an *inhA* mutation. For the 20 INH-resistant isolates with no *katG* or *inhA* promoter mutation, the first 135 codons of the *inhA* structural gene were sequenced, and all were wild type. On its own, the detection of an *inhA* mutation was 16.5% sensitive and 100% specific (Table 1). When the results for both *katG* and *inhA* are considered together, the assay sensitivity improves from 85.4% to 90.6% and the specificity remains 100% (Table 1).

Ethambutol and *embB*. EMB targets *embB*-encoded arabinosyl transferase and thereby inhibits arabinogalactan synthesis. Mutations within a region of *embB* designated the ERDR were analyzed, since mutations in this region have been reported in 50 to 60% of EMB-resistant isolates. In this study, a total of 154 isolates were resistant to 5 μg/ml EMB, of which 124 (80.5%) had at least one mutation within the region of *embB* examined. Among the 160 EMB-susceptible isolates, 27 had an *embB* mutation. In total, 158 *embB* nSNPs were identified, consisting of 16 unique mutations within 11 different codons (see Table S5 in the supplemental material). One silent mutation occurred at leucine residue 355. Eighty-five (53.8%) of the *embB* single mutations occurred within codon 306 (ATG), resulting in methionine being replaced by valine (GTG), isoleucine (ATA), or leucine (CTG) in 53, 29, and 3 isolates, respectively. All 53 isolates with an *embB* Met306Val substitution were EMB resistant, as were the 3 isolates with Met306Leu. In contrast, 5 (17.2%) of the 29 isolates harboring the Met306Ile allele were EMB susceptible. Other substitutions found within EMB-susceptible isolates include Ala313Val (two isolates) and Asp354Ala (two isolates). The GAC-to-GCC codon change producing the Asp354Ala substitution also occurred in nine EMB-resistant isolates. Twenty-two isolates had an *embB* mutation, resulting in the substitution of alanine (GCG) for glutamic acid (GAG) at residue 378 (Glu378Ala), and in 18 of those isolates this was the only *embB* mutation. Sixteen of the isolates with only the EmbB Glu378Ala substitution were EMB susceptible, and nine of those were pansusceptible. Four EMB-resistant isolates had a Glu378Ala substitution in conjunction with another *embB* mutation: two with Met306Val and one each with Met306Ile and Asp328Tyr. Two isolates with only the Glu378Ala substitution in EmbB were EMB resistant. Our finding that 20 (91%) of the isolates with an *embB* Glu378Ala substitution either were EMB susceptible or had an additional *embB* mutation to explain the isolate's resistance leads us to conclude that this

mutation represents a naturally occurring lineage marker and does not confer EMB resistance. In fact, 18 of the 22 isolates harboring the mutation were determined to belong to the IndoOceanic lineage. If the Glu378Ala substitution is treated as a naturally occurring polymorphism, the detection of *embB* nSNPs for predicting EMB phenotypic results exhibits a sensitivity of 78.6% and specificity of 93.1% (Table 1).

Pyrazinamide and *pncA*. PZA is a prodrug that requires conversion to its active metabolite by the *pncA*-encoded enzyme, pyrazinamidase. Some mutations within *pncA* are associated with loss of enzyme activity and both are, in turn, highly correlated with PZA resistance. The entire *pncA* open reading frame and additional upstream sequence were analyzed for 249 of the study isolates (see Table S1 in the supplemental material). DNA sequencing was not completed for the 60 WHO isolates and failed to produce data of acceptable quality for five CDC isolates after three attempts each. PZA susceptibility and *pncA* sequencing results were available for 192 isolates, of which 65 were PZA resistant. Among the resistant isolates, 55 (84.6%) possessed a *pncA* mutation and 10 retained the wild-type sequence, while in the case of the 127 PZA-susceptible isolates, 109 (88.6%) lacked a *pncA* mutation, while 15 harbored mutations (see Table S6 in the supplemental material). Considering all 246 isolates with *pncA* sequence results, 116 possessed a *pncA* mutation. These mutations consisted of 79 unique alterations distributed throughout the gene and regulatory region. The majority (84/116; 72.4%) of these were nSNPs, but insertions ($n = 10$ isolates), deletions ($n = 9$), and nonsense ($n = 6$), synonymous ($n = 3$), and putative regulatory ($n = 4$) mutations were also identified. The most frequent mutation was a Thr47Ala change, found in five isolates. Three isolates had a synonymous mutation at serine residue 65.

Three methods of PZA susceptibility testing were used in this study, with 13 isolates being tested by the Bactec and agar proportion methods, 7 by the Bactec and MGIT methods, and the remainder by one of the three techniques. No isolates were tested by both the MGIT and agar proportion methods. When the test sensitivity and specificity for the three methods are calculated separately and independently, the values are 89.2% and 94.4%, respectively, for the Bactec method ($n = 112$), 83.3% and 83.9%, respectively, for the agar proportion methods ($n = 49$), and 93.3% and 79.4%, respectively, for the MGIT method ($n = 51$). Two of the isolates tested by both the Bactec and MGIT methods had discordant results, while the results for all those tested by both the Bactec and agar proportion methods were concordant. In the two discrepant isolates, the Bactec result was used in calculating the test accuracy values. Cumulatively, the identification of nSNPs within *pncA* exhibited a sensitivity and specificity of 84.6% and 85.8%, respectively (Table 1).

Fluoroquinolones and *gyrA*. Resistance to the FQs has been attributed to mutations within subunit A of DNA gyrase (encoded by *gyrA*), which prevent the drugs from effectively binding the gyrase (44). In this study, we analyzed the QRDR of *gyrA*, which is known to house the majority of mutations associated with resistance (33). DST data were available for CIP (314 isolates) and OFX (245 isolates). A total of 98 isolates were resistant to either CIP ($n = 94$) or OFX ($n = 83$), including 79 resistant to both drugs. Four isolates were resistant only to OFX, 14 isolates were resistant to CIP and did not have DST results for OFX, and 1 isolate was resistant to CIP but susceptible to OFX. Out of the total number of resistant isolates, 80 (90%) were found to harbor mutations within the QRDR of *gyrA* (see Table S7 in the supplemental material). The most common mutations were observed at codon 94 ($n = 49$), where the wild-type aspartic acid (GAC) was replaced with a glycine (D94G; $n = 26$), an alanine (D94A; $n = 11$), an asparagine (D94N; $n = 10$), or a tyrosine (D94Y; $n = 2$) (Table 2). The Asp94Gly mutation was also found in two combinations, once with an Ala90Val mutation and once with a Ser91Pro mutation. In addition, 26 isolates harbored the Ala90Val mutation, and of these, 25 were FQ resistant, while 1 was susceptible. Other mutations associated with the FQ-resistant isolates included Asp89Asn ($n = 1$), Asp89Gly ($n = 1$), Gly88Cys ($n = 2$), and Ser91Pro ($n = 2$). The two mutations at codon 89 were not previously reported in the literature. The remaining 18 FQ-resistant isolates were determined to be wild type for the QRDR of *gyrA*.

Of the 161 isolates determined to be susceptible to both CIP and OFX, one contained the resistance-associated Ala90Val mutation and four harbored the Thr80Ala (ACC → GCC) mutation, which has previously been reported to provide weak FQ hypersusceptibility (2). All other susceptible isolates were determined to be wild type for the QRDR of *gyrA*. When the genotypic data for *gyrA* were compared with the drug susceptibility results for the FQ, the sensitivity and specificity values were determined to be 81.6% and 97.7%, respectively (Table 1).

Amikacin and *rrs*. Mutations associated with resistance to AMK are located within *rrs*, which encodes the ribosomal 16S rRNA (1). In this study, we analyzed a 516-bp region of *rrs* that contains the most common resistance-associated mutations. Genotypic and phenotypic data were available for 313 study isolates, where 70 (22.4%) were resistant to 4 μg/ml amikacin and 243 (77.6%) were AMK susceptible. All of the AMK-resistant isolates were found to be cross-resistant to either KAN (32; 45.7%) or KAN and CAP (38; 54.3%). Sixty-three (90%) of the resistant isolates were found to have *rrs* mutations, including 61 with A1401G resistance-associated mutations, 1 with the resistance-associated T1322C and A1401G combination of mutations, and 1 with a G1484T resistance-associated mutation (see Table S8 in the supplemental material).

Of the 243 AMK-susceptible isolates, 240 were determined to be wild type for *rrs*. One isolate contained a C1402T mutation, which has been reported to be associated with AMK susceptibility, and 2 isolates (representing 1 WHO duplicate) contained a previously unreported A1196G mutation (18) (see Table S8 in the supplemental material). Final comparison of the genotypic and phenotypic data produced a sensitivity value of 90% and a specificity value of 98.8% (Table 1). If the values are calculated using only the A1401G mutation, the sensitivity decreases minimally to 87.1% and the specificity increases to 100%.

Kanamycin and *rrs* and *eis*. Known mechanisms of KAN resistance are caused by mutations in *rrs* or the promoter region of *eis* which prevent the drug from inhibiting translation (15, 45). In this study, a 516-bp region of the open reading frame of *rrs* and the promoter region of *eis* were analyzed, as they are known to contain resistance-associated mutations. Of the 314 isolates, 111 (35.4%) were resistant to KAN, and 96

(86.5%) of these isolates contained mutations within the *rrs* (64; 57.7%) or *eis* (32; 28.8%) regions of interest (see Tables S8 and S9 in the supplemental material). None of the isolates contained mutations within both loci. The most frequent *rrs* mutation was A1401G (62; 96.9%), which was observed in 61 isolates as a single mutation and in 1 isolate with the previously unreported T1322C substitution (Table 2). The isolate harboring the A1401G and T1322C double mutation was also resistant to amikacin but susceptible to capreomycin. Other *rrs* polymorphisms included the resistance-associated C1402T ($n = 1$) and G1484T ($n = 1$) mutations (18). Mutations within the promoter region of *eis* included the resistance-associated G(−10)A (14; 43.8%), C(−14)T (10; 31.3%), G(−37)T (5; 15.6%), and C(−2)A-C(−14)T combination mutations. One isolate harbored a C(−12)T mutation, which has been associated with susceptibility to KAN (45).

A total of 203 (64.7%) isolates were KAN susceptible, of which 195 (96.1%) were wild type for both the *rrs* and *eis* loci, and 8 (3.9%) harbored mutations within either *rrs* ($n = 2$) or *eis* ($n = 6$). None of the isolates had mutations within both loci. The two isolates with mutations within *rrs* represented one WHO duplicate and contained a previously unreported A1196G mutation. The seven *eis* mutations included four promoter polymorphisms and three amino acid substitutions. The promoter mutations included three susceptibility-associated C(−12)T substitutions and one resistance-associated G(−10)A substitution. The open reading frame mutations were observed in two isolates, with one harboring a silent Pro90Pro substitution and the other having a combination of a silent Ser48Ser substitution and a Met100Thr polymorphism.

Upon comparison of the genotypic and phenotypic data, *rrs* was found to be 57.7% sensitive and 99% specific, while *eis* was 28.8% sensitive and 97.0% specific (excluding the silent mutations) (Table 1). Upon combination of the data sets for both loci, the sensitivity value increased to 86.5% and the specificity value changed to 96.1% (Table 1).

Capreomycin and *rrs* and *tlyA*. Resistance to CAP is associated with mutations within *rrs* and *tlyA* genes in *M. tuberculosis* (19, 34). In this study, a 516-bp region of *rrs* and the entire open reading frame of *tlyA* were analyzed, as these areas are known to house the majority of resistance-associated mutations. Of the 314 total clinical isolates in this study, 69 (22%) were resistant to CAP, including 29 susceptible to AMK or KAN. Of the 69 resistant isolates, 42 (60.9%) contained mutations within *rrs* ($n = 38$) or *tlyA* ($n = 7$), 3 contained mutations in both genes, and 27 (39.1%) were wild type for both loci (see Tables S8 and S10 in the supplemental material). The most frequent *rrs* polymorphism was the A1401G mutation, which was found in 34 (89.5%) of the resistant isolates (Table 2). Other *rrs* mutations included two novel A1196G mutations (WHO duplicate isolates), one resistance-associated C1402T mutation, and one resistance-associated G1484T mutation (18). Mutations within the *tlyA* open reading frame included two amino acid substitutions at codon 236, Asp236Lys, one previously unreported Asp57His mutation, two GC insertions at nucleotide 202 (WHO duplicate isolates), and two Gly196Glu mutations (WHO duplicate isolates).

A total of 245 (78%) study isolates were CAP susceptible, with 31 (12.7%) containing mutations in either *rrs* or *tlyA* and 214 (87.3%) being wild type for both loci. The mutations harbored by CAP-susceptible strains were located in either *rrs* or *tlyA*, with no overlap between the two genes. For isolates with polymorphisms within *rrs*, a total of 28 (90.3%) isolates had the A1401G mutation, which occurred as single events in 27 isolates and together with a T1322C substitution in 1 isolate. The *tlyA* mutations included a Gly195Asp mutation, a Leu164Ser mutation, and a silent mutation at codon 28.

Comparison of the drug susceptibility data and the genotypic data for CAP (*rrs* only) produced a sensitivity value of 55.1% and a specificity value of 88.6% (Table 1). Data for *tlyA* without combination with *rrs* resulted in a 10.1% sensitivity and a 98.8% specificity (excluding the silent mutation) (Table 1). Upon combination of *rrs* and *tlyA* data, the overall sensitivity and specificity values change to 60.9% and 87.3%, respectively (Table 1).

Cross-resistance among the second-line injectable drugs AMK, KAN, and CAP is well documented and also supported by this study (1, 18, 34, 36). Of the 111 KAN-resistant isolates, 38 (34.2%) were KAN monoresistant, 38 (34.2%) were cross-resistant with both AMK and CAP, 32 (28.8%) were cross-resistant with only AMK, and 2 (1.8%) were cross-resistant with only CAP. All of the AMK-resistant isolates were cross-resistant with either KAN or KAN and CAP, but none were resistant to only AMK. Of the 69 CAP-resistant isolates, only 29 (42%) were CAP monoresistant, although the remaining 40 were cross-resistant with either KAN or KAN and AMK. The 38 KAN-, AMK-, and CAP-resistant isolates included 35 with mutations within *rrs*, 1 with a combination *rrs* and *tlyA* mutations, and 3 that were wild type for all loci (see Table S11 in the supplemental material). The isolates with resistance-associated *rrs* mutations included 34 with A1401G mutations and 1 with a G1484T mutation. The *tlyA* mutation resulted in the Asp57His amino acid change in combination with an A1401G *rrs* substitution (see Table S11 in the supplemental material). The 32 KAN- and AMK-resistant isolates included 28 with *rrs* mutations (27 with A1401G single mutations and 1 with the T1322C and A1401G combination), 1 with an *eis* mutation [C(−14)T], and 3 of the wild type (see Table S11 in the supplemental material). Lastly, the two isolates resistant to both KAN and CAP were wild type for both *eis* and *tlyA*, while one contained a C1402T mutation within *rrs* (see Table S11 in the supplemental material).

DISCUSSION

The results of this study demonstrate the utility of detection of mutations associated with drug resistance to rapidly and accurately determine the drug susceptibility of *M. tuberculosis* complex isolates. This study also demonstrates the advantages of DNA sequencing over methods that use DNA probes to detect deviation from the wild-type sequence or a limited set of specific mutations, since several specific mutations identified in this study were not associated with drug resistance. Each resistance-associated locus examined has its own unique properties which must be taken into consideration when the molecular drug sequencing results are interpreted.

The molecular mechanism of resistance to RIF is the most completely understood of the resistance mechanisms of all the drugs used in the treatment of tuberculosis, and it has been

well established that mutations within the RRDR of *rpoB* occur in 95% or more of RIF-resistant isolates. Within the RRDR, three specific mutations predominate (Ser531Leu, His526Tyr, and Asp516Val), presumably because these mutations minimally impact the fitness of the bacilli (16, 29). In this study, 140 of the 174 (80.5%) RIF-resistant isolates harbored one of these three *rpoB* mutations. However, many other RRDR mutations have been described, including 25 within our study (see Table S3 in the supplemental material). Since these other mutations are far less frequently encountered, their association with RIF resistance has not been well established. Some of these *rpoB* mutations, including Leu511Pro, Asp516Tyr, His526Leu/Ser, and Leu533Pro, are associated with discordant susceptibility test results (37). In a previous study, 11 isolates harboring these mutations were classified RIF susceptible by either the Bactec 460 TB or Bactec 960 MGIT system, while only 1 was susceptible by the proportion method on Löwenstein-Jensen medium and only 2 were susceptible on Middlebrook 7H10 agar (37). The authors proposed that these mutations could confer low-level but clinically relevant RIF resistance (37). Others have considered isolates harboring the Leu533Pro substitution to be susceptible (17, 25). In this study, the His526Asn, His526Leu, and Leu533Pro mutations were identified in eight susceptible and eight resistant isolates. Four of the eight resistant isolates contained other resistance-associated *rpoB* mutations. These data weakly support the association of these mutations with low-level resistance. However, further studies are required to definitively characterize their role in RIF resistance. The relative rarity of these mutations may be partially attributable to selection bias, as most studies have examined primarily or exclusively RIF-resistant isolates. With increased use of genotypic methods to diagnose RIF resistance, these mutations may be more frequently observed, and subsequently, their clinical and epidemiological significance may be better understood. The specificity of DNA sequencing as a diagnostic tool will increase, should future research establish that certain RRDR mutations do not impart a clinically relevant level of RIF resistance. The possibility that such mutations exist has important ramifications for methods which detect the presence of any mutation and not the mutation of interest.

The mode of action of isoniazid, though extensively investigated, remains incompletely understood. Several different loci are known to be involved in resistance, especially *katG* and *inhA*. Mutations in codon 315 of *katG* and the *inhA* promoter are proven mechanisms of INH resistance. None of the INH-susceptible isolates in this study had a mutation in either *katG* codon 315 or the *inhA* promoter, making the test 100% specific. Nineteen of the INH-resistant isolates were wild type at both these locations and also in the region of the *inhA* structural gene where resistance-associated mutations have been reported (data not shown) (3, 4). Although mutations in other loci, such as *ndh* and *ahpC*, may account for the INH-resistant phenotype in some of these isolates, we chose not to sequence these loci because there are very limited data on their role in INH resistance.

Until recently, the significance of mutations within the *M. tuberculosis embB* gene, especially those at codon 306, had been controversial because such mutations had been reported in both EMB-susceptible and -resistant isolates (11, 21, 30). Allelic exchange experiments have now convincingly shown that mutations at *embB* codons 306, 406, and 497 confer EMB resistance (27, 28, 32). In those earlier reports of EMB-susceptible isolates harboring *embB* mutations, the drug susceptibility results were presumably inaccurate. Conventional culture-based methods of EMB susceptibility testing are notoriously problematic (14). The allelic exchange studies demonstrated that the mutants with Met306Val and Met306Leu mutations had EMB MICs well above the critical concentration used for drug susceptibility testing, and indeed, we found that all of those isolates with only a Met306Val ($n = 53$) or Met306Leu ($n = 3$) substitution were EMB resistant. In contrast, MICs for the mutants with the Met306Ile mutation were only modestly higher than the critical concentration, a confounding circumstance that could result in false-susceptible EMB testing results. This expectation is supported by our finding that 5 of the 29 isolates possessing only a Met306Ile substitution were EMB susceptible.

The third most frequent *embB* substitution in this study was Glu378Ala. This was the only *embB* mutation in 22 isolates, of which 16 were EMB susceptible. This substitution was previously reported in a single isolate (26) and in combination with an Asp299Glu substitution in two isolates (31), and in both reports, the isolates were characterized to be EMB resistant. The probable explanation for the paucity of reports of this mutation is that prior studies have examined primarily EMB-resistant strains. In this study, we examined a large number of EMB-susceptible and pansusceptible isolates to more accurately assess the drug susceptibility phenotype of the mutations identified. Of the 22 isolates, 18 were determined to belong to the IndoOceanic lineage by spoligotyping. Therefore, our data indicate that the mutation resulting in the Glu378Ala substitution is a naturally occurring polymorphism and possible lineage marker that does not confer EMB resistance. Functional genetic analysis of this mutation is needed to definitively validate this conclusion.

We identified 20 different *embB* mutations or combination of mutations, including the previously discussed Glu378Ala mutation. This diversity of mutations gives DNA sequencing a definite advantage over hybridization-based methods, particularly given that a putative polymorphism occurs within the ERDR. Although further functional genetic data are needed to better define the phenotype of additional *embB* mutations, complementing inherently problematic culture-based DST with DNA sequencing would substantially improve diagnostic accuracy.

The presence of a mutation within *pncA* has been shown to correlate well with PZA resistance (22), and a diverse and widely distributed array of *pncA* mutations has been reported (29). We also found a diverse group of *pncA* mutations that correlated well with PZA resistance. Conventional DST of PZA is challenging and problematic due to the poor growth of *M. tuberculosis* under the acidic conditions (pH 5.5 to 6.0) required for optimal drug activity (13, 20). The good correlation between the presence of a *pncA* mutation and PZA resistance makes DNA sequencing of this gene a very useful adjunctive diagnostic test. Unfortunately, the tremendous diversity of reported *pncA* mutations and the lack of any predominant mutations represent a substantial limitation. Without strong statistical evidence linking specific mutations with PZA resistance, further experimental evidence is needed to

determine which *pncA* mutations confer resistance. Mindful of these limitations, the use of *pncA* sequencing in conjunction with phenotypic PZA testing could aid in determining PZA susceptibility. Additional studies to characterize the phenotype of specific *pncA* mutations through the determination of MIC values or functional genetics would greatly enhance the diagnostic value of *pncA* sequencing.

The FQs are known to be effective against *M. tuberculosis* by targeting and inhibiting the essential bacterial enzyme DNA gyrase (44). It has been well established that mutations within *gyrA*, which encodes subunit A of the DNA gyrase, are often associated with resistance (33, 35, 40). The most common resistance-associated mutations within *gyrA* are found within the QRDR at codons 94 and 90, as was the case with our study (35, 40). In addition to the known mutations, we discovered the novel Asp89Asn and Asp89Gly substitutions. Because these mutations have not been previously reported, their association with resistance is unknown. Lastly, 18 FQ-resistant isolates did not harbor any mutations within the *gyrA* locus. These isolates could be explained by mutations within the *gyrB* locus (unexamined in this study) or by unknown mechanisms of FQ resistance.

The FQ-susceptible isolates harbored primarily wild-type *gyrA* loci, with few exceptions. One isolate contained the resistance-associated Ala90Val mutation, and repeat sequencing confirmed the discordant result. However, repeat DST was unable to be completed due to the lack of subsequent growth from the original isolate, leaving the possibility of irreproducible phenotypic results. In addition, four FQ-susceptible isolates harbored the Thr80Ala mutation, which has been reported as providing a strain with increased susceptibility, and are therefore not discordant (2).

Because resistance to one of the second-line injectable drugs is part of the definition of XDR TB, the establishment of rapid assays for detecting resistance to these drugs is of particularly high importance. All of these drugs are known to target the bacterial ribosome, and mutations within the 16S rRNA, encoded by *rrs*, are well established as being associated with second-line drug resistance. Most of the resistance-associated mutations within *rrs* are found within a 516-bp region of the gene. In this study, *rrs* mutations associated with AMK-resistant isolates included a large number of expected A1401G mutations as well as a resistance-associated G1484T mutation. Both of these mutations have been previously described as playing a central role in AMK resistance (18). In addition to the established mutations, we also discovered a previously unreported T1322C and A1401G combination of mutations in an isolate resistant to AMK, KAN, and CAP. Since the T1322C mutation has not been previously identified, its role in aminoglycoside resistance is unclear, both as a lone mutation and in combination with the A1401G substitution. However, its presence in an isolate that is 100% resistant to both KAN and AMK suggests that it is associated with aminoglycoside resistance. We did not detect any resistance-associated mutations in the vast majority of AMK-susceptible isolates; however, one contained the C1402T mutation for AMK susceptibility and two harbored novel A1196G SNPs (18). Because the substitution was found only in susceptible isolates, it is unlikely that it is associated with AMK resistance.

Alterations within the *rrs* locus also play a significant role in KAN resistance, with the 65 detected RRDR mutations accounting for 60% of substitutions within KAN-resistant isolates (1, 15, 34). The detected A1401G, C1402T, and G1484T mutations have all been well established as resistance-associated substitutions, leaving the novel A1401G and T1322C mutation combination to be the only allele with unknown implications (18). Two study isolates that were both AMK and KAN susceptible harbored previously unknown A1196G mutations, suggesting that these substitutions do not confer resistance.

In addition to *rrs*, mutations within the promoter region of *eis*, which encodes an aminoglycoside acetyltransferase, are associated with low-level KAN resistance (45). In our survey, we identified 38 *eis* mutations, including 3 previously unreported substitutions. The most common previously reported resistance-associated *eis* mutations included the G(−10)A, C(−14)T, and G(−37)T polymorphisms (45). Our study supports these findings, as they were also the most prominent in our KAN-resistant isolates (see Table S9 in the supplemental material). Also in agreement with the previous study was our finding of the C(−12)T mutation in a KAN-resistant isolate and three KAN-susceptible isolates (see Table S9 in the supplemental material). These data support the proposed hypothesis that this mutation may result in an MIC value close to the recommended critical concentration for testing KAN susceptibility (45). As a result, the C(−12)T mutation is considered a polymorphism with little predictive value for KAN resistance. It is also of note that we identified one G(−10)A mutation in a KAN-susceptible isolate from the 2008 WHO proficiency testing panel. Because WHO isolates were not subjected to repeat testing, this result remains unconfirmed and may be explained by undetected low-level KAN resistance. Lastly, our detection of the Ser48Ser and Met100Thr mutation combination within the open reading frame of *eis* is novel, and the potential effects of open reading frame mutations on KAN resistance are unknown. However, *eis*-mediated resistance is due to promoter mutations that alter *eis* expression levels, and the impact of open reading frame mutations on KAN resistance would potentially require a separate mechanism.

Although the combination of mutations within *rrs* and *eis* resulted in a relatively high specificity (86.5%), 15 KAN-resistant isolates did not harbor mutations in either gene. Two of these 15 isolates were determined to be KAN susceptible upon repeat drug susceptibility testing, suggesting that they may display a level of resistance close to the current testing concentration. Two of the wild-type isolates were duplicates of each other from the 2008 WHO panel, thereby representing one isolate whose DST result was not confirmed with repeat testing. In addition, two isolates were susceptible to 5 μg/ml KAN with subsequent repeat testing and were therefore no longer considered discordant. The nine remaining isolates were confirmed to be discordant by repeat testing and exhibited a range of resistance levels, suggesting that there remains at least one unknown mechanism for KAN resistance (data not shown).

Similar to KAN, resistance to the macrocyclic polypeptide CAP is associated with mutations within two separate loci: *rrs* and *tlyA* (19, 34, 36). Polymorphisms within the *rrs* locus affect CAP binding to the ribosome, while those within *tlyA* prevent necessary methylation of rRNA, rendering their ribosomes CAP resistant (19). Common *rrs* mutations associated with

CAP resistance include the prominent A1401G mutation and the C1402T and G1484T mutations. Our study detected all of these polymorphisms as well as two novel A1196G mutations, whose role in aminoglycoside resistance is currently unknown. Previous studies have often demonstrated a strong correlation between the A1401G mutation and CAP resistance (10, 18, 39). However, other reports have found the same mutation in isolates determined to be CAP susceptible (5, 9, 12, 38). In this study, we detected a large number of A1401G mutations in both CAP-resistant and CAP-susceptible isolates (see Table S8 in the supplemental material). The mutations were verified by repeat DNA sequencing; however, the drug susceptibility testing result was found to vary widely upon repeat testing. In fact, select isolates were retested up to five times, with different results obtained for each testing cycle (data not shown). These data suggest that the CAP MIC value for isolates harboring the A1401G mutation may be very close to the current recommended critical concentration for testing CAP susceptibility on 7H10 agar. Previous studies may not have detected this variation due to relatively smaller study sizes, limited numbers of susceptible isolates, or variation in CAP drug susceptibility testing methods.

Mutations within *tlyA* are uncommon, possibly due to limited and local usage of CAP to treat *M. tuberculosis* infections (5, 12, 39). Polymorphisms within *tlyA* in our study were less common than those within *rrs*, with the total number accounting for 13% of study mutations associated with CAP resistance. The addition of *tlyA* mutation data to *rrs* mutation data in our study increased our sensitivity and specificity for CAP resistance from 55.1% and 88.6% to 60.9% and 87.3%, suggesting that *tlyA* currently plays a minor role in the detection of CAP resistance and could be used sparingly as a resistance marker, as long as usage of the drug remains low.

Cross-resistance between the second-line injectables has been well documented and is further supported by our study data (5, 18, 38). As expected, the ribosomal *rrs* mutation A1401G was the most common polymorphism associated with cross-resistance between KAN, AMK, and CAP, as well as KAN and AMK (see Tables S8 and S11 in the supplemental material). One isolate, resistant to both KAN and AMK, harbored an A14041G substitution in combination with a T1322C mutation (see Tables S8 and S11 in the supplemental material). Because the latter polymorphism has not been previously described and resistance to both drugs can be explained by the *rrs* substitution, association of this novel mutation with resistance to either drug is unclear. It is worth noting that none of the isolates were resistant only to AMK, suggesting that detection of AMK resistance may be a greater indication of second-line cross-resistance. Lastly, although there was a high level of agreement between our genotypic and phenotypic data for injectable cross-resistance, some isolates were wild type for all resistance-associated alleles (see Table S11 in the supplemental material). This result suggests that there is still at least one mechanism of second-line injectable drug cross-resistance that has yet to be discovered.

There is a current need for the development of rapid molecular tests that detect mutations associated with drug resistance in strains of *M. tuberculosis*. Conventional culture-based techniques are technically demanding and have long turnaround times. Molecular techniques, such as the Sanger-based DNA sequencing method presented here, provide specific and prompt strain resistance data that aid in the development of appropriate antituberculosis treatment regimens. The development of genetic assays requires the verification of molecular data with respect to conventional drug susceptibility data. Here we completed a comprehensive survey of nine loci known to harbor mutations associated with resistance to both first-line and second-line anti-TB drugs. The resultant genotypic data set was compared with drug susceptibility data to determine sensitivity and specificity values for each locus (Table 1). These values served to verify the new CDC MDDR service and have the potential to aid in the development of other, in-house molecular assays. Developed genetic tests will inevitably produce more rapid results for drug-resistant isolates, which will lead to faster identification of MDR and XDR strains, more tailored treatment regimens, and a reduction in the transmission of TB.

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