Frequency and Distribution of Tuberculosis Resistance-Associated Mutations between Mumbai, Moldova, and Eastern Cape

S. B. Georghiou, a M. Seifert, a D. Catanzaro, b R. S. Garfein, a F. Valafar, d V. Crudu, d C. Rodrigues, d T. C. Victor, f A. Catanzaro, a T. C. Rodwell a

Department of Medicine, University of California, San Diego, La Jolla, California, USA; a University of Arkansas, Fayetteville, Arkansas, USA; a Biological and Medical Informatics Research Center, San Diego State University, San Diego, California, USA; a Microbiology and Morphology Laboratory, Institute of Phthisiopneumology, Chisinau, Moldova; a Department of Microbiology, P. D. Hinduja Hospital and Medical Research Centre, Mumbai, India; a Division of Molecular Biology and Human Genetics, Stellenbosch University, Stellenbosch, South Africa.

Molecular diagnostic assays, with their ability to rapidly detect resistance-associated mutations in bacterial genes, are promising technologies to control the spread of drug-resistant tuberculosis (DR-TB). Sequencing assays provide detailed information for specific gene regions and can help diagnostic assay developers prioritize mutations for inclusion in their assays. We performed pyrosequencing of seven Mycobacterium tuberculosis gene regions (katG, inhA, ahpC, rpoB, gyrA, rrs, and eis) for 1,128 clinical specimens from India, Moldova, and South Africa. We determined the frequencies of each mutation among drug-resistant and -susceptible specimens based on phenotypic drug susceptibility testing results and examined mutation distributions by country. The most common mutation among isoniazid-resistant (INHr) specimens was the katG 315ACC mutation (87%). However, in the Eastern Cape, INHr specimens had a lower frequency of katG mutations (44%) and higher frequencies of inhA (47%) and ahpC (10%) promoter mutations. The most common mutation among rifampin-resistant (RIFr) specimens was the rpoB 331TTG mutation (80%). The mutation was common in RIFr specimens in Mumbai (83%) and Moldova (84%) but not the Eastern Cape (17%), where the 516GTC mutation appeared more frequently (57%). The most common mutation among fluoroquinolone-resistant specimens was the gyrA 94GGC mutation (44%). The rrs 1401G mutation was found in 84%, 84%, and 50% of amikacin-resistant, capreomycin-resistant, and kanamycin (KAN)-resistant (KANr) specimens, respectively. The eis promoter mutation 12T was found in 26% of KANr and 4% of KAN-susceptible (KANs) specimens. Inclusion of the ahpC and eis promoter gene regions was critical for optimal test sensitivity for the detection of INH resistance in the Eastern Cape and KAN resistance in Moldova. (This study has been registered at ClinicalTrials.gov under registration number NCT02170441.)

In 2014, an estimated 9.6 million people developed tuberculosis (TB), and 1.5 million people died of their infection (1). Although global TB incidence rates have fallen an average of 1.5% per year since 2000, the rise of drug-resistant TB (DR-TB) globally has complicated TB control efforts (1). The World Health Organization (WHO) estimates that as many as 1 in every 20 new, active TB infections is now drug resistant (1). One of the major roadblocks in combating this growing problem has been the lack of diagnostic technology for DR-TB. Current growth-based culture and drug susceptibility testing (DST) methods can take several weeks to months to yield results (2). While waiting on culture and DST methods can take several weeks to months to yield results (2). While waiting on culture results, physicians are forced to treat their patients empirically, adjusting treatment regimens only once DST results become available. As a result, many undiagnosed DR-TB patients are being given medications that are ineffective, which amplifies resistance, increases the risk of mortality, and increases the risk of transmitting DR-TB infections in the community.

Rapid molecular diagnostic assays for DR-TB have the potential to curb this spread of resistance by shortening the time to TB diagnosis and effective treatment. These technologies identify and characterize DR-TB infections based upon the presence or absence of known resistance-conferring mutations in the Mycobacterium tuberculosis genome (3–5). Unfortunately, the vast majority of rapid molecular DR-TB diagnostic tests, including line probe and microarray assays, rely on a closed set of mutations for resistance detection (6–8). The decision of which mutations to include in these assays is generally based upon the global frequencies of known resistance-associated mutations and the strength of the association between these mutations and phenotypic drug resistance to corresponding antituberculosis drugs of interest. The relationship between phenotypic drug resistance and mutation status is not always 100%, however, and although recent systematic reviews have given us a better idea of the relationship between particular mutations and phenotypic drug resistance (9–12), data are still lacking for rare and novel mutations. Furthermore, little is known about regional distributions of TB resistance-associated mutations, which could affect the performance of molecular diagnostic assays implemented globally.

Global studies providing phenotypic as well as complete genotypic sequence information for DR-TB clinical specimens are necessary in order to further inform the development of molecular diagnostic assays. Unlike most rapid DR-TB molecular diagnostic
assays, such as the line probe assays and real-time amplification-based assays, sequencing assays yield long sequencing reads, allowing detailed genetic analysis of diverse clinical specimens. Sequencing technologies have the additional advantage of being open assays, meaning that they can be easily modified to accommodate our evolving knowledge of the genetic basis of TB phenotypic drug resistance. The Global Consortium for Drug-Resistant Tuberculosis Diagnostics (GCDD) conducted a large, multisite study evaluating the diagnostic performance of a modified pyrosequencing diagnostic assay for DR-TB in three diverse clinical environments (13) and in doing so generated sequencing data for epidemiologically different populations of DR-TB patients. This study presents the frequencies and distributions of all identified resistance-associated mutations and considers the implications of these findings for the expected performance of rapid molecular diagnostic assays in diverse clinical environments.

MATERIALS AND METHODS

Study population. Three epidemiologically diverse clinical sites (Chisinau, Moldova; Port Elizabeth, South Africa; and Mumbai, India) were selected for this study. In India, patients were enrolled at P. D. Hinduja Hospital and Medical Research Centre, the main DR-TB referral center for the city of Mumbai and the state of Maharashtra. In Moldova, TB patients were enrolled in four regional TB hospitals: two in Chisinau, one in Voronceni, and one in Balti. All patient samples were processed at the Phthisiopneumology Institute in Chisinau, a scientific research, medical consultation, and training center that is the central unit of the Moldovan National TB Control Program. In Port Elizabeth, patients were enrolled at one regional hospital and six primary health care facilities spread throughout the region. Newly presenting TB patients over 5 years of age were eligible for the study if they were known to be acid-fast bacillus smear positive or were suspected of having active pulmonary TB and having one or more reasons to be considered to have DR-TB and provided informed consent for the study. Of the eligible patient population, 52 patients were excluded for an inability to provide 7.5 ml of sputum (n = 35) or for other or unknown reasons (n = 17). A total of 1,128 patients with risk factors for DR-TB were enrolled from 24 April 2012 to 27 June 2013 (14).

Drug susceptibility testing. Mycobacterial Growth Indicator Tube 960 (MGIT960) cultures were performed. MGIT DST results served as the phenotypic reference standard in our study. All specimens were tested for resistance to isoniazid (INH), rifampin (RIF), moxifloxacin (MFX), ofloxacin (OFX), amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) according to the manufacturer’s protocols and using critical concentrations recommended by the WHO and reported previously for MGIT-based drug susceptibility testing at the time of our study: 0.1 μg/ml for INH, 1.0 μg/ml for RIF, 2.0 μg/ml for OFX, 0.25 μg/ml for MFX, 1.0 μg/ml for AMK, 2.0 μg/ml for CAP (15, 16), and 2.5 μg/ml for KAN (17, 18). For the purposes of this analysis, specimens resistant to either MFX or OFX via MGIT DST were considered fluoroquinolone (FQ) resistant (FQ’). Specimens that were not phenotypically resistant to MFX and OFX were considered FQ susceptible (FQ”). All specimens with discordant phenotypic results for the two FQs (n = 11) are presented in Table S1 in the supplemental material.

DNA extraction, molecular targets, and PCR. DNA was extracted from each decontaminated, concentrated sputum sample (sediment) by heating the cell suspensions in a water bath at 100°C (14, 19). Our pyrosequencing assay included eight reactions: one to identify M. tuberculosis and seven to detect mutations in drug resistance-associated gene regions (see Table S2 in the supplemental material). All primers used in this study, other than the rrs primers, are specific for M. tuberculosis and do not show cross-reactivity with other TB species (19). We associated INH resistance with mutations in the rhlP promoter (positions −4 to −23), the inhA promoter (positions −4 to −20), and katG (codons 312 to 316). RIF resistance was associated with mutations within rpoB (codons 507 to 533), FQ resistance was associated with mutations other than the natural polymorphism 95ACC in gyrA (codons 88 to 95) (20), and resistance to injectable drugs (KAN, AMK, and CAP) was associated with mutations in rrs (positions 1397 to 1406). PCR primers for these gene regions were previously described (19). Upon completion of this study, eis promoter (positions −5 to −47) sequencing capability was added to our platform, ensuring specificity for M. tuberculosis via hybridization analysis for cross-reactivity to other TB species (see Table S2 in the supplemental material). Mutations in the eis promoter, in addition to rrs mutations, were associated with KAN resistance (21). PCR master mixes were prepared, and amplification reactions were carried out for all targets, as previously reported (19).

Pyrosequencing. Pyrosequencing was performed according to the manufacturer’s procedures and modified for sequencing of mutations associated with DR-TB, as described previously (4, 19). We utilized the PyroMark Q96 1D system (Qiagen, Valencia, CA) to perform pyrosequencing on the nine targets detailed above, sequencing two parts of rpoB in two separate reactions. Variants were identified automatically following pyrosequencing by using Identifire software (Qiagen, Valencia, CA) (12, 22). Testing of samples that did not provide sequencing queries that 100% matched library sequences was repeated in duplicate. Samples that still did not provide a confirmatory sequence, and samples for which contradictory hits were obtained, were deemed genotypically indeterminate.

Cumulative mutation frequencies. Cumulative mutation frequencies were established for every mutation identified in our study across all clinical sites. Mutation frequencies were determined for all relevant drug-resistant and -susceptible specimens for which sequence data were obtained. For each gene region of interest (i.e., “only katG”), only those specimens with both a relevant phenotypic DST result and a sequencing result for the given region(s) of interest were included in mutation sensitivity and specificity calculations.

Mutation distributions between clinical sites. Site-specific mutation frequencies were also established for every mutation identified in our study. The number of times that a mutation appeared in relevant drug-resistant and -susceptible specimens was summarized for each gene region of interest for each clinical site. As with cumulative mutation frequencies, only those mutations with both sequencing data and a phenotypic DST result for each relevant drug were considered when establishing site-specific mutation frequencies. Mutation frequencies among drug-resistant specimens between the different clinical sites are presented as bar graphs.

Human research conduct. Our study, registered with ClinicalTrials.gov (registration number NCT02170441), was reviewed and approved by the Institutional Review Board of the University of California, San Diego, and by the Institutional Review Boards of the participating institutions at the three study sites. All participants provided written informed consent. Participation did not alter the standard of care.

RESULTS

DST results. Nine hundred fourteen (81%) of the 1,128 patients enrolled in the study provided M. tuberculosis culture-positive pulmonary sputum samples. Of these 914 samples, 768 (84%) were smear positive. Of the remaining 214 samples, 1 was culture contaminated, and 213 were M. tuberculosis culture negative. Seven of the 914 culture-positive samples either did not have MGIT DST performed or did not yield results for any of the anti-tuberculosis drugs evaluated. Of the original 1,128 patients, 454 (40%) had multidrug-resistant TB (MDR-TB), and 80 (7%) had extensively drug-resistant TB (XDR-TB) (data not shown).

Cumulative mutation frequencies. Cumulative mutation frequencies are presented in Tables 1 to 4.

(i) Isoniazid resistance-associated mutations. The katG 315ACC mutation was the most common INH resistance-asso-
ciated mutation identified in this study. This mutation was found in 480 specimens across all sites, including 139 (29%) cooccurrences with \(\text{inhA}\) or \(\text{ahpC}\) promoter mutations. Overall, the 315ACC mutation was found in 473 (87%) INH-resistant (INHr) specimens and 133 (24%) INH-sensitive (INHs) specimens. Within the \(\text{inhA}\) promoter, the −15T mutation was most commonly identified, appearing in 135 specimens: 133 (24%) INHr specimens and 2 (1%) INHs specimens. The −15T mutation cooccurred with \(\text{katG}\) or \(\text{ahpC}\) promoter mutations in 110 (81%) of these 135 specimens. Within the \(\text{ahpC}\) promoter, the −10A mutation was most commonly identified, appearing in seven (1%) INHr specimens, including four cooccurrences with \(\text{katG}\) and \(\text{inhA}\) promoter mutations. Twenty-six (5%) of the total 516 INHr specimens with sequencing reads for both \(\text{katG}\) and \(\text{inhA}\) promoter mutations were wild-type isolates. Interestingly, \(\text{rpoB}\) mutations were also identified in 10 Rifr specimens (Table 2). Together, all of the mutations identified in the \(\text{rpoB}\) gene region encompassing codons 507 to 533 helped to explain 97% of the phenotypic RIF resistance in our study.

(ii) Rifampin resistance-associated mutations. The \(\text{rpoB}\) 531TTG mutation was the most common RIF resistance-associated mutation identified in this study, appearing in 360 (80%) Rifr specimens across all sites. Fourteen (4%) of the total 389 Rifr specimens with sequencing reads for both \(\text{rpoB}\) gene regions were found to be wild-type isolates. Interestingly, \(\text{rpoB}\) mutations were also identified in 10 Rifr specimens (Table 2). Together, all of the mutations identified in the \(\text{rpoB}\) gene region encompassing codons 507 to 533 helped to explain 97% of the phenotypic RIF resistance in our study.

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specimens with *rrs* and *eis* sequencing reads were wild-type isolates. After inclusion of the *eis* promoter gene target, the combined sensitivity of all identified resistance-associated mutations in the seven *M. tuberculosis* gene regions sequenced in this study was 84 to 97% for DR-TB drugs.

**Mutation distributions.** (i) **Isoniazid resistance-associated mutations.** Differences were noted in the frequencies of mutations associated with INH resistance between the three clinical sites (Fig. 1; see also Table S3 in the supplemental material). The *katG* 315ACC mutation was present in 89% of INHr samples in India and 92% of INHr samples in Moldova but only 44% of INHr specimens in South Africa. A higher percentage of South African INHr specimens had mutations in the *inhA* (47%) or *ahpC* promoter (10%) gene region than seen in India or Moldova.

### Table 2: Cumulative frequencies of all mutations among *Mycobacterium tuberculosis* specimens resistant or susceptible to rifampin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Observed mutation(s) by gene location</th>
<th>No. of RIF&lt;sup&gt;r&lt;/sup&gt; specimens sequenced</th>
<th>No. of RIF&lt;sup&gt;r&lt;/sup&gt; specimens with mutation</th>
<th>No. of RIF&lt;sup&gt;s&lt;/sup&gt; specimens with mutation</th>
<th>Frequency of mutation among RIF&lt;sup&gt;r&lt;/sup&gt; specimens (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency of mutation among RIF&lt;sup&gt;s&lt;/sup&gt; specimens (%)&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
<td>516GTC</td>
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<td>262</td>
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<td>5.2</td>
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<td></td>
<td>526GAC</td>
<td>450</td>
<td>266</td>
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<td></td>
<td>531TTG</td>
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<td>266</td>
<td>9</td>
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<td>533CCG</td>
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<td></td>
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</tr>
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<td>266</td>
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<tr>
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* Frequency of mutation among RIF<sup>r</sup> specimens was calculated as the number of RIF<sup>r</sup> specimens with the mutation/number of RIF<sup>r</sup> specimens sequenced.

* Frequency of mutation among RIF<sup>s</sup> specimens was calculated as the number of RIF<sup>s</sup> specimens with the mutation/number of RIF<sup>s</sup> specimens sequenced.

### Table 3: Cumulative frequencies of all mutations among *Mycobacterium tuberculosis* specimens resistant or susceptible to fluoroquinolones

<table>
<thead>
<tr>
<th>Gene</th>
<th>Observed mutation(s) by gene location</th>
<th>No. of FQ&lt;sup&gt;r&lt;/sup&gt; specimens sequenced</th>
<th>No. of FQ&lt;sup&gt;r&lt;/sup&gt; specimens with mutation</th>
<th>No. of FQ&lt;sup&gt;s&lt;/sup&gt; specimens with mutation</th>
<th>Frequency of mutation among FQ&lt;sup&gt;r&lt;/sup&gt; specimens (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency of mutation among FQ&lt;sup&gt;s&lt;/sup&gt; specimens (%)&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>94GCC and 95ACC</td>
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* Frequency of mutation among FQ<sup>r</sup> specimens was calculated as the number of FQ<sup>r</sup> specimens with the mutation/number of FQ<sup>r</sup> specimens sequenced.

* Frequency of mutation among FQ<sup>s</sup> specimens was calculated as the number of FQ<sup>s</sup> specimens with the mutation/number of FQ<sup>s</sup> specimens sequenced.
though Moldova had a large number of INH\textsuperscript{r} specimens with the \textit{inhA} – 15T mutation (46%), almost all of these mutations cooccurred with a \textit{katG} codon 315 mutation.

\textbf{(ii) Rifampin resistance-associated mutations.} Differences were also observed in the frequencies of mutations associated with RIF resistance between the three sites (Fig. 2; see also Table S4 in the supplemental material). The \textit{rpoB} 531TTG mutation was present in 83 to 84% of RIF\textsuperscript{r} specimens in India and Moldova but only 17% of RIF\textsuperscript{r} specimens in South Africa. Instead, the \textit{rpoB} 516GTC mutation was found more frequently (57%) among RIF\textsuperscript{r} specimens in South Africa.

\textbf{(iii) Fluoroquinolone resistance-associated mutations.} Unlike the mutations associated with INH or RIF resistance, none of the \textit{gyrA} resistance-associated mutations identified in our study were found in more than 50% of the FQ\textsuperscript{r} specimens in any clinical site (Fig. 3; see also Table S5 in the supplemental material). The \textit{gyrA} 94GGC mutation was the mutation most frequently identified among FQ\textsuperscript{r} specimens in India (46%) and South Africa (46%), but the mutation was identified in only 6% of FQ\textsuperscript{r} specimens in Moldova. Instead, the 90GTG (19%), 94GCC (19%), and 91CCG (13%) mutations were more commonly identified among FQ\textsuperscript{r} specimens evaluated in Moldova. The 90GTG mutation was also identified in 66 (26%) FQ\textsuperscript{r} specimens in India.

\textbf{(iv) Injectable resistance-associated mutations.} The \textit{rrs} 1401G mutations showed notable differences in its frequencies among injectable-resistant specimens between the clinical sites (Fig. 4; see also Table S6 in the supplemental material). The mutation appeared in 85 to 94% of the injectable-resistant specimens evaluated in India and South Africa, although it was less common in Moldova, appearing in 33%, 7%, and 40% of Moldovan AMKR, KAN\textsuperscript{r}, and CAP\textsuperscript{r} specimens, respectively. Mutations in the \textit{eis} promoter were more common than \textit{rrs} mutations among the KAN\textsuperscript{r} specimens evaluated in Moldova. The \textit{eis} promoter –12C/T mutation was found in 37 (53%) KAN\textsuperscript{r} specimens in Moldova. The –14C/T, –10G/A, and –37G/T mutations were also identified in eight (11%) KAN\textsuperscript{r} specimens in Moldova. These \textit{eis} promoter mutations were also found in India (nine specimens), although they appeared in a mix of KAN\textsuperscript{r} and KAN\textsuperscript{s} specimens. The –15C/G mutation was

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Gene & Mutation & INJ & No. of INJ\textsuperscript{r} specimens sequenced & No. of INJ\textsuperscript{r} specimens with mutation & No. of INJ\textsuperscript{s} specimens with mutation & Frequency of mutation among INJ\textsuperscript{r} specimens (%) & Frequency of mutation among INJ\textsuperscript{s} specimens (%) \\
\hline
\hline
\textit{rrs} only & 1401G & AMK & 73 & 61 & 83.6 & 0.7 \\
& & KAN & 121 & 61 & 50.4 & 0.7 \\
& & CAP & 70 & 59 & 84.3 & 1.0 \\
\hline
\textit{eis} only & –12C/T & AMK & 78 & 1 & 1.3 & 8.4 \\
& & KAN & 141 & 37 & 26.2 & 4.2 \\
& & CAP & 75 & 1 & 1.3 & 8.4 \\
\hline
\textit{rrs} only & –10G/A & AMK & 78 & 0 & 0.0 & 0.8 \\
& & KAN & 141 & 5 & 3.5 & 0.1 \\
& & CAP & 75 & 0 & 0.0 & 0.8 \\
\hline
\textit{rrs} only & –14C/T & AMK & 78 & 1 & 1.3 & 0.5 \\
& & KAN & 141 & 3 & 2.1 & 0.5 \\
& & CAP & 75 & 0 & 0.0 & 0.9 \\
\hline
\textit{rrs} only & –37G/T & AMK & 78 & 0 & 0.0 & 0.5 \\
& & KAN & 141 & 3 & 2.1 & 0.1 \\
& & CAP & 75 & 0 & 0.0 & 0.5 \\
\hline
\textit{rrs} only & –10G/C & AMK & 78 & 0 & 0.0 & 0.4 \\
& & KAN & 141 & 0 & 0.0 & 0.4 \\
& & CAP & 75 & 0 & 0.0 & 0.4 \\
\hline
\textit{rrs} only & –15C/G & AMK & 78 & 0 & 0.0 & 0.1 \\
& & KAN & 141 & 1 & 0.7 & 0.0 \\
& & CAP & 75 & 0 & 0.0 & 0.1 \\
\hline
\textit{rrs} or \textit{eis} with no mutations & Wild type & AMK & 73 & 12 & 16.4 & 99.3 \\
& & KAN & 121 & 60 & 49.6 & 99.3 \\
& & CAP & 70 & 11 & 15.7 & 99.0 \\
\hline
\textit{rrs} or \textit{eis} with no mutations & Wild type & AMK & 71 & 9 & 12.7 & 88.4 \\
& & KAN & 119 & 18 & 15.1 & 93.4 \\
& & CAP & 68 & 9 & 13.2 & 88.0 \\
\hline
\end{tabular}
\caption{Cumulative frequencies of all mutations among \textit{Mycobacterium tuberculosis} specimens resistant or susceptible to amikacin, kanamycin, and/or capreomycin}
\end{table}
identified in one (2%) KANr specimen in India. Notably, no  

eis promoter mutations were found in any South African specimens  
in this study, and no  
eis promoter mutations cooccurred with  

rrs mutations.

**DISCUSSION**

Large-scale sequencing studies remain critical for DR-TB molecular  
diagnostic assay development, as they enable assay developers  
to prioritize resistance-associated mutations for optimal diagnostic  
performance and to predict diagnostic assay performance globally.  
We conducted a large, multisite DR-TB sequencing study to  
determine the frequencies of all significant resistance-conferring mutations across three diverse clinical sites and characterized differences in the distributions of the mutations between the sites. Notably, inclusion of the  

ahpC and  
eis promoter gene regions was found to be critical for optimal assay sensitivity for INH resistance detection in the Eastern Cape and for KAN resistance detection in Moldova.

**Isoniazid resistance-associated mutations.** The most common  

katG,  

inhA, and  

ahpC promoter mutations in our study were identified at frequencies similar to those reported previously (11,  

23,  

24). However, our finding of 315ACC mutations among INHr specimens (2.8%) was unexpected. The  
katG 315ACC mutation has been associated with an INH MIC of 3 to >16 μg/ml by DST using liquid medium (25–28) and was shown to confer INH resistance at 5 μg/ml in an allelic exchange study (29). Therefore, it is highly unlikely that any  

M. tuberculosis specimens with the  
katG 315ACC mutation would have an INH MIC below the critical concentration used in this study (0.1 μg/ml). The seven discordant results observed were likely false-positive results, resulting from PSQ failure rather than DST error. Of the  

inhA promoter mutations identified in our study, only the  

-15T and  

-17T mutations were identified in INHr specimens, both at low frequencies of 0.7%. These frequencies are slightly higher than the reported global frequencies of 0.0 to 0.3% (11), but  

inhA promoter mutations generally convey lower levels of resistance to INH than do  
katG mutations (MICs ranging from 0.1 to >16.0 μg/ml) (30–32), and so it is possible that the four study specimens with these mutations had MIC values close to the INH critical concentration and were interpreted as being INHr by liquid culture. Although  

inhA and  

ahpC mutations occurred independently in a small proportion (1 to 6%) of the  
thREE alleles identified in one (2%) KANr specimen in India. Notably, no  
eis promoter mutations were found in any South African specimens in this study, and no  
eis promoter mutations cooccurred with  

rrs mutations.

**FIG 1** Frequency of mutations associated with INH resistance identified among INHr specimens by clinical site.

**FIG 2** Frequency of mutations associated with RIF resistance identified among RIFr specimens by clinical site.
their inclusion as molecular markers of INH resistance in our diagnostic assay.

Important differences were seen in the distributions of INH resistance-associated mutations between the clinical sites. Although ahpC promoter mutations were identified in only a low proportion (3.1%) of the INH-resistant specimens in our study, these mutations appeared in a substantial proportion (10.5%) of the INH-resistant specimens in the Eastern Cape. A previous study conducted in KwaZulu-Natal found a similar frequency of ahpC promoter mutations among INH-resistant isolates (12.6%), although they all cooccurred with katG mutations (33). In contrast, our study identified many ahpC mutations without cooccurring katG or inhA promoter mutations. Previous studies suggested that the selection of ahpC mutations occurs only after the accumulation of katG mutations (34), yet our study finds ahpC mutations to be independent markers of resistance in different patient populations, similar to the findings reported previously by Silva et al. (24). Despite ahpC promoter mutations being rare globally (5.4% of all INH-resistant specimens) and often cooccurring with katG mutations (11, 34), they may play a significant role in explaining regional phenotypic INH resistance patterns. If these mutations are excluded from molecular diagnostic tests, then these tests may experience significant decreases in sensitivity in certain geographical regions. The inclusion of the ahpC promoter in our assay in the Eastern Cape, for example, was critical to our detection of INH resistance in this region. This finding also has important implications for the performance of other DR-TB molecular diagnostic assays, such as the Hain MTBDRplus line probe assay, which does not include the ahpC promoter mutations (35). If this assay were used to detect INH resistance in our South African study population, ~8.6% of INH-resistant strains would have been missed without the addition of ahpC promoter mutations. Adding this gene target to DR-TB molecular diagnostic assays could improve assay sensitivity for INH resistance detection both regionally and globally.

Rifampin resistance-associated mutations. The most common rpoB mutations identified, 531TTG (80.0%) and 516GTC (5.2%), appeared across study sites at frequencies comparable to those previously reported for a set of RIF-resistant M. tuberculosis isolates in a multisite study (68.8% and 6.8%, respectively) (4). All other mutations in the 81-bp rpoB RIF resistance-determining region appeared to explain the lower (14.8%) but still significant proportion of RIF resistance in this study, confirming that the inclusion of this entire gene region in rapid molecular diagnostic assays is important to best predict phenotypic RIF resistance. Interestingly, resistance-associated rpoB mutations were also identified in 10

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![FIG 3](https://example.com/figure3.png)

**FIG 3** Frequency of the most common mutations associated with injectable resistance identified among AMK<sup>+</sup>, KAN<sup>+</sup>, and/or CAP<sup>+</sup> specimens by clinical site.

![FIG 4](https://example.com/figure4.png)

**FIG 4** Frequency of the most common mutations associated with injectable resistance identified among AMK<sup>+</sup>, KAN<sup>+</sup>, and/or CAP<sup>+</sup> specimens by clinical site.
RIF specimens, suggesting that they were poor predictors of phenotypic RIF resistance, as opposed to previous findings (36). This discrepancy is likely related to the complexities of the liquid culture-based DST that we used to determine RIF resistance for these low-MIC mutants. Both liquid medium- and solid medium-based DST methods use WHO-endorsed critical concentrations of RIF that are ideally equivalent; however, for these particular rpoB mutations resulting in low-MIC RIF resistance, the results are consistently RIF with liquid medium (MGIT960) and RIF with solid medium. Therefore, these mutants likely have MICs near the WHO-recommended RIF critical concentration established for liquid-based DST (37, 38). It is critical to understand the relationship between these mutations and the levels of phenotypic resistance that they confer in order to accurately interpret the results of molecular diagnostic assays that rely upon these genetic markers to predict phenotypic RIF resistance.

The frequencies of the rpoB 531TTG mutation were much higher in India (83%) and Moldova (84%) than in the Eastern Cape (17.4%). The inclusion of the 516GTC mutation (56.5% of RIF specimens in the Eastern Cape) appeared to be more important for molecular test performance in this region. Most molecular diagnostic assays, including the Hain MTBDRplus and Cepheid GeneXpert MTB/RIF assays, include the 81-bp RIF resistance-determining region of the rpoB gene region sequenced in this study and therefore would be expected to detect all major mutations that we identified between the three sites.

Fluoroquinolone resistance-associated mutations. Mutations conferring resistance to the FQ compounds were most often identified at gyrA codons 90 (25.5%) and 94 (60.4%), in line with previously reported findings (9, 39). As with rpoB, mutations spread throughout the gyrA quinolone resistance-determining region contributed to the prediction of phenotypic FQ resistance, confirming the need to include this entire gene region in molecular diagnostic tests. Our study also provided information regarding the rare gyrA mutations 88GCC and 88TGC, which were found exclusively in FQ’ specimens. Although these mutations contributed only 1.8% to the prediction of phenotypic FQ resistance in this study, their reliability as FQ resistance markers may support their inclusion in molecular diagnostic assays such as the Hain MTBDRsl assay (40).

No large differences were identified between clinical sites in regard to the frequencies of various mutations in the gyrA gene region, and no single mutation appeared in more than 46% of FQ’ specimens in any site. The inclusion of the quinolone resistance-determining region of the gyrA gene spanning codons 88 to 95 was adequate to detect the majority (64 to 96%) of FQ resistance in each of the three sites, and observed variations in the sensitivity of the assay for FQ resistance detection between the sites were similar to those reported previously (41–43).

Injectable resistance-associated mutations. The rrs 1401G mutation was identified in injectable-resistant and -susceptible specimens in the range of reported global frequencies (56 to 78% and 0 to 7%) (12). eis promoter mutations were also identified among injectable-resistant specimens within the range of global estimates (0 to 22%) (12), but many eis promoter mutations also appeared in KAN’ specimens. In order to investigate this discrepancy, 15 KAN’ specimens with eis mutations were subjected to repeat phenotypic KAN DST at the critical concentration (2.5 μg/ml), with all repeated MGIT DST reactions being run in duplicate. Eleven specimens (73%) were KAN’ in at least one of the two duplicate DST runs, but five of these results were discordant between the two runs, suggesting a possible mixture of KAN’ and KAN’ isolates in the sample that then grew out as either resistant or susceptible when cultured. The other four specimens were KAN’ in both DST runs, indicating that these mutants had MICs below the tested KAN critical concentration. While eis promoter mutations have been well documented to confer only low-level KAN resistance (21, 44), recent studies have found M. tuberculosis eis mutants to have broad KAN MIC ranges (0.625 to 32 μg/ml) via liquid-based DST methods (45, 46). As such, the eis promoter mutations identified in our study may have had MICs around the critical concentration. Although eis promoter mutations do not appear to be reliable predictors of KAN resistance above 2.5 μg/ml, this is probably as much a reflection of the uncertainties around our understanding of KAN’ phenotypes and the critical concentrations that we use for measuring resistance as the uneven expression of resistance in these mutants. Despite these limitations, the inclusion of these mutations in our diagnostic assay helped to explain 86% of the phenotypic KAN resistance in our study, compared to 50% based solely upon the rrs 1401G mutation.

Our initial pyrosequencing assay, based solely upon the detection of mutations at the rrs gene region at codon 1401, predicted 84.6 to 93.9% of injectable resistance in India and the Eastern Cape. However, the mutation appeared in only a few injectable-resistant specimens in Moldova. The inclusion of the eis promoter resulted in a large gain in platform sensitivity for KAN resistance detection in Moldova (7% to 79%), in line with data from previously reported studies, as eis promoter mutations have been documented to occur at a high frequency in countries that were once part of the former Soviet Union, due to heavy reliance upon KAN in TB treatment regimens (47, 48). Interestingly, no eis mutations cooccurred with rrs mutations. Previous studies documented eis promoter mutation selection prior to rrs mutation selection (47), as rrs mutations have already evolved high-level resistance to KAN and would not benefit from the addition of eis promoter mutations. Additionally, no eis promoter mutations were found in the Eastern Cape. This finding is important, as technologies lacking the eis promoter gene target would show high sensitivity for KAN resistance detection in our South African population but low sensitivity for KAN resistance detection in our Indian and Moldovan populations.

Limitations. All results presented in this study should be considered specific to our study populations in the large cities of Mumbai, Chisinau, and Port Elizabeth and not necessarily the countries of India, Moldova, and South Africa. It is therefore possible that the observed variations in mutation frequencies between the sites may be representative of localized DR-TB outbreaks or the persistence of endemic drug-resistant clones in these locales. However, our results highlighted a diversity of DR-TB strains with unique genetic combinations, suggesting that the vast majority of studied infections were not clonal. Our results are noteworthy if they reflect true regional differences rather than local outbreaks, as these genetic variations will affect the performance of rapid molecular diagnostic assays in larger regions. Additionally, although the mutations that we identified provide a larger picture of the genetic basis of phenotypic antituberculosis drug resistance, they do not represent a complete genetic profile of the DR-TB specimens evaluated in this study. The inclusion of other gene regions, such as novel katG and fabG1 mutations recently associated with INH
resistance (32), may further increase the sensitivity of rapid molecular diagnostic assays for DR-TB detection. Additional sequencing studies investigating other genes and gene regions, such as *slyA* and *gudB* mutations and their association with injectable resistance (12), will be necessary to identify the genetic basis of drug resistance for the 4 to 16% of genetically wild-type, drug-resistant specimens in this study.

Conclusions. We conducted a large, multisite DR-TB sequencing study and found a wide diversity of mutations that varied in frequency between three diverse clinical sites. Altogether, the 46 resistance-associated mutations identified in seven gene targets were sufficient to detect 84 to 97% of XDR-TB phenotypes in this study. Inclusion of the *ahpC* and *es* promoter gene regions was critical for optimal test sensitivity for INH resistance detection in the Eastern Cape and KAN resistance detection in Moldova. The identification of *rpoB* and *es* promoter mutations in a large number of RIF- and KAN*-specimens in this study emphasizes the need for future studies to address discordant phenotypic results for these low-MIC mutations and verify the clinical relevance of these mutations. These findings may help diagnostic assay developers to prioritize gene regions and mutations for inclusion in their assays, although DR-TB diagnostic assays that include all knowledge-resistance-associated mutations will likely remain the best option for optimal sensitivity of molecular diagnostic assays for DR-TB detection.

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