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Impact of \textit{gyrB} and \textit{eis} Mutations in Improving Detection of Second-Line-Drug Resistance among \textit{Mycobacterium tuberculosis} Isolates from Georgia

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\textbf{ABSTRACT} The country of Georgia has a high burden of multi- and extensively drug-resistant tuberculosis (XDR-TB). To evaluate whether mutations in \textit{gyrB} and \textit{eis} genes increased the sensitivity of detection of phenotypic resistance to ofloxacin and kanamycin or capreomycin compared to use of the first-generation MTBDRsl assay alone, which tests for mutations in \textit{gyrA} and \textit{rrs} genes, a retrospective study of stored \textit{Mycobacterium tuberculosis} isolates was performed. All isolates underwent DNA sequencing of resistance-determining regions. Among 112 \textit{M. tuberculosis} isolates with DNA extraction data, targeted sequencing was successfully performed for each gene as follows: for \textit{gyrA}, 98\% sensitivity; for \textit{gyrB}, 96\%; for \textit{rrs}, 93\%; for the \textit{eis} gene and its promoter, 93\%. The specificity and hence the positive predictive value of \textit{gyrA} and \textit{gyrB} mutations for detecting ofloxacin resistance were 100\%. The addition of \textit{gyrB} mutations increased the sensitivity of phenotypic ofloxacin resistance detection by 13\% (75\% to 88\%). All \textit{rrs} resistance-conferring mutations were A1401G, and this mutation had low sensitivity (40\% and 18\%) and high specificity (95\% and 100\%) in predicting phenotypic capreomycin and kanamycin resistance, respectively. The \textit{eis} C-14T mutation increased the sensitivity of phenotypic kanamycin resistance detection by 9\% (18\% to 27\%) and was found solely in kanamycin phenotypic resistance isolates. Our data showed that the inclusion of \textit{eis} C-14T and \textit{gyrB} mutations in addition to \textit{rrs} and \textit{gyrA} mutations improves the sensitivity of detection of phenotypic ofloxacin and kanamycin resistance, respectively.

\textbf{KEYWORDS} Georgia, DNA sequencing, \textit{eis}, \textit{gyrB}, drug resistant

The year of 2016 marked a critical juncture in the battle against tuberculosis (TB) as new goals outlined in the Sustainable Developmental Goals (SDGs) and the End TB Strategy called for accelerated action to reduce the incidence of the disease by at least 90\% over the next 2 decades (1, 2). A major threat to these goals is the continued emergence of multidrug-resistant (MDR) and, even more concerning, extensively drug-resistant (XDR) TB (3). The 2016 World Health Organization (WHO) report noted a continued high global incidence of MDR TB at 580,000 new cases and further resistance in the form of XDR strains at a rate of approximately 10\% among those with MDR. XDR TB has now been documented in 105 countries (1). Outcomes for patients with MDR and XDR TB remain poor due in part to difficulties in detection of drug resistance and lack of optimal antituberculosis medications (4).

An area that offers great promise in improving drug-resistant-TB management is the introduction of rapid molecular diagnostic tests. Modeling work suggests that the
implementation of rapid diagnostics and subsequent initiation of effective treatment can have a major effect on reducing the burden of drug-resistant TB (5, 6). The endorsement and rollout of a line-probe assay (LPA) (Genotype MTBDRplus; HainLife-science) and the Xpert MTB/RIF (Cepheid) assay have been a testament to the benefits of rapid testing (1). The Xpert assay and LPA have both been shown to drastically reduce the time to detection of MDR TB, in most cases by over a month compared to culture and drug susceptibility testing (DST) (7, 8). Additionally, we previously demonstrated that implementation of the MTBDRplus assay led to a significantly reduced time to culture conversion and implementation of proper infection control measures among patients with MDR TB in the country of Georgia (9). An accurate rapid molecular test for detection of fluoroquinolones and injectable agents (kanamycin, amikacin, and capreomycin) is needed to realize similar benefits for patients with XDR TB.

The MTBDRsV1 assay detects mutations in the gyrA gene (coding for subunit A of DNA gyrase) and the rrs gene (coding for16S rRNA) as a means to determine phenotypic fluoroquinolone and injectable-drug resistance, respectively. A recent review on the performance of the MTBDRsV1 assay concluded that the test is able to accurately rule in drug resistance but is precluded from ruling out resistance due to low sensitivity, especially for kanamycin resistance (10). We reported a similar lack of sensitivity in detected XDR-TB in using the MTBDRsV1 LPA in Georgia. We found suboptimal performance for detection of phenotypic drug resistance to ofloxacin (81% sensitivity), capreomycin (57%), kanamycin (27%), and XDR TB (41%) compared to that of traditional phenotypic DST (11).

More-recent work has found that the inclusion of tests for additional mutations in the gyrB and eis genes may improve the detection of phenotypic resistance to tofluoroquinolones and kanamycin, respectively (12–15), and those results have also led to the development of the MTBDRsV2 assay, which includes tests for gyrB and eis promoter mutations (16). We sought to evaluate whether we could improve the molecular detection of phenotypic ofloxacin, kanamycin, and capreomycin resistance using the MTBDRsV1 assay through the inclusion of tests for additional mutations in gyrB and eis promoter genes, including those found in the newer MTBDRsV2 assay, utilizing targeted DNA sequencing. Georgia continues to be inflicted with high rates of XDR TB, and the results of this study are expected to help efforts to develop an accurate rapid test for XDR TB that will work in our setting and in other, similar settings. An accurate rapid test for XDR TB would help pave the way for timely introduction of effective treatment.

RESULTS

Among the 112 Mycobacterium tuberculosis isolates with DNA extraction, targeted sequencing was successfully performed for each gene as follows: for gyrA, 98% (110/112) sensitivity; for gyrB, 96% (107/112); for rrs, 93% (104/112); for the eis gene and its promoter, 93% (104/112). The primer sequences for amplification of the four gene targets in the gyrA, gyrB, and rrs genes and in the eis gene and its promoter are listed in Table 1.

Sixteen (14%) of the 112 M. tuberculosis isolates were phenotypically resistant to ofloxacin. No gyrA or gyrB mutations were identified by either MTBDRsV1 testing or targeted DNA sequencing in the phenotypically susceptible isolates (Table 2). Among the 16 ofloxacin-resistant isolates, 14 (88%) had a mutation in either gyrA (n = 11) or gyrB (n = 5) according to both tests, and in one case there was the lack of binding to the gyrA wild-type 2 (ΔWT2) probe by the MTBDRsV1 test. Two isolates had both a gyrA and gyrB mutation, and another two isolates had no mutations identified (Table 2). The addition of any gyrB mutation increased the sensitivity of ofloxacin resistance detection from 75% to 88%. The specificity and hence the positive predictive value (PPV) determined for gyrA and for detection of ofloxacin resistance were 100% (Table 3). gyrA mutations D94G (n = 6) and A90V (n = 5) were the most common mutations (Table 2). One isolate had a gyrA mutation identified by the MTBDRs assay but not by sequencing, while another isolate had a gyrA mutation found by sequencing but not by the
MTBDR\textit{sl} assay. The overall agreement between molecular detection of \textit{gyrA} and a combination of \textit{gyrA} and \textit{gyrB} mutations for phenotypic ofloxacin resistance was high (kappa values of 0.84 and 0.92, respectively) (Table 3).

Among the 112 \textit{M. tuberculosis} isolates, 15 (13\%) and 63 (56\%) were phenotypically resistant to capreomycin and kanamycin, respectively (Tables 4 and 5). With the exception of one susceptible isolate with an \textit{rrs} mutation (C1443G), the only \textit{rrs} mutation detected by the MTBDR\textit{sl} assay and targeted sequencing was A1401G (Tables 4 and 5). In regard to capreomycin, the \textit{rrs} A1401G mutation was found in 6 of 15 (40\%) and 4 of 97 (4\%) phenotypically resistant and susceptible isolates, respectively (Table 4). Any \textit{eis} promoter mutations were found in isolates phenotypically susceptible (25/97, 26\%) and resistant (4/15, 27\%) to capreomycin with similar prevalences. The presence of an \textit{rrs} mutation had low sensitivity (6 of 15, 40\%) and high specificity (90 of 95, 95\%) in predicting phenotypic capreomycin resistance (Table 6). There were 2 isolates with

### TABLE 1 PCR primers\textsuperscript{a}

<table>
<thead>
<tr>
<th>Drug(s) and gene or promoter</th>
<th>Sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofloxacin \textit{gyrA}</td>
<td>F → 5’TGG GAT CTA ACC GGT TGA CAT-3’</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>R → 5’TGG CTT CCG TCT ACC TCA T3’</td>
<td></td>
</tr>
<tr>
<td>\textit{gyrB}</td>
<td>F → 5’TAC ACC GAG GTA AAA TGG TT-3’</td>
<td>695</td>
</tr>
<tr>
<td></td>
<td>R → 5’CTG AAT GCC GTC TCT CTT GT-3’</td>
<td></td>
</tr>
<tr>
<td>Kanamycin, capreomycin \textit{rrs}</td>
<td>F → 5’TAC GCT GAC TAA GAA CCC TAC CAC G3’</td>
<td>731</td>
</tr>
<tr>
<td></td>
<td>R → 5’TAC ATG CTT GCC CAC AGT TCG ATC-3’</td>
<td></td>
</tr>
<tr>
<td>\textit{eis} gene</td>
<td>F → 5’TCT GGT ACC GCC CAG AGT TCG CGT AC-3’</td>
<td>814</td>
</tr>
<tr>
<td></td>
<td>R → 5’TCC GCC TGG TCT GTG CAC ACT-3’</td>
<td></td>
</tr>
<tr>
<td>\textit{eis} promoter</td>
<td>F → 5’TGC GAG TCT TGG CAC ACT-3’</td>
<td>787</td>
</tr>
<tr>
<td></td>
<td>R → 5’TCC GCG GTC TCC CTT CGT CGC AC-3’</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}bp, base pairs; F, forward; R, reverse.

### TABLE 2 Comparison of analysis of \textit{gyrA} and \textit{gyrB} mutations with phenotypic ofloxacin drug susceptibility testing results in \textit{Mycobacterium tuberculosis} isolates (\(n = 112\))\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Ofloxacin phenotypic DST result and no. of isolates</th>
<th>MTBDR\textit{sl} assay result and mutation(s)\textsuperscript{b}</th>
<th>\textit{gyrA} sequencing result</th>
<th>\textit{gyrB} sequencing result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible ((n = 96))</td>
<td>Susceptible</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>92</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>WT</td>
<td>NA</td>
</tr>
<tr>
<td>Resistant ((n = 11))</td>
<td>Resistant</td>
<td>(\Delta\textit{WT2} + \textit{MT1})</td>
<td>A90V</td>
</tr>
<tr>
<td>2</td>
<td>(\Delta\textit{WT2} + \textit{MT1})</td>
<td>A90V</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>(\Delta\textit{WT3} + \textit{MT3C})</td>
<td>D94G</td>
<td>WT</td>
</tr>
<tr>
<td>1\textsuperscript{c}</td>
<td>(\Delta\textit{WT4} + \textit{MT1})</td>
<td>A90V</td>
<td>A543T</td>
</tr>
<tr>
<td>1</td>
<td>\textit{MT3C}</td>
<td>WT</td>
<td>N538T</td>
</tr>
<tr>
<td>1\textsuperscript{c}</td>
<td>(\Delta\textit{WT2})</td>
<td>WT</td>
<td>R485C</td>
</tr>
<tr>
<td>Resistance ((n = 5))</td>
<td>Susceptible</td>
<td>A90V</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>A90V</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>R485H</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>A543V</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}DST, drug susceptibility testing; \(\Delta\), indicated lack of wild-type band; WT, wild type; MT, mutation; NA, not available.

\textsuperscript{b}\textit{gyrA} gene region or mutation analyzed: WT2, positions 89 to 93; WT3, positions 92 to 97; MT1, mutation A90V; MT3C, mutation D94G.

\textsuperscript{c}Data represent \textit{M. tuberculosis} isolates with a mutation in both \textit{gyrA} and \textit{gyrB}.
Of the 63 *M. tuberculosis* isolates with phenotypic resistance to kanamycin, only 11 were detected either by the presence of an *rrs* mutation (10) or, in one case of detection by the MTBDR<sub>sl</sub> assay, by a lack of binding to *rrs* wild-type probe 1 (ΔWT1). There were 3 isolates with an *rrs* mutation found by the MTBDR<sub>sl</sub> assay but not by sequencing, and there was 1 isolate with an *rrs* mutation found by sequencing but not by the MTBDR<sub>sl</sub> assay. The *rrs* A1401G mutation was not found in any isolates phenotypically suscep-

### TABLE 3 Performance parameters of *gyrA* and *gyrB* mutations in detecting any resistance to ofloxacin compared to conventional drug susceptibility testing (reference standard)<sup>a</sup>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ofloxacin value(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>gyrA</em> (n = 112)</td>
</tr>
<tr>
<td>No. of isolates showing:</td>
<td></td>
</tr>
<tr>
<td>True susceptibility</td>
<td>96</td>
</tr>
<tr>
<td>True resistance</td>
<td>12</td>
</tr>
<tr>
<td>False susceptibility</td>
<td>4</td>
</tr>
<tr>
<td>False resistance</td>
<td>0</td>
</tr>
<tr>
<td>% sensitivity (95% CI)</td>
<td>75 (54–96)</td>
</tr>
<tr>
<td>% specificity</td>
<td>100</td>
</tr>
<tr>
<td>% PPV</td>
<td>100</td>
</tr>
<tr>
<td>% NPV (95% CI)</td>
<td>96 (92–100)</td>
</tr>
<tr>
<td>Kappa (95% CI)</td>
<td>0.84 (0.68–0.99)</td>
</tr>
</tbody>
</table>

<sup>a</sup>gyrA mutations were determined by either MTBDR<sub>sl</sub>V1 testing or genetic sequencing. CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

<sup>b</sup>No *gyrB* genetic sequencing results were available for 5 isolates.

an *rrs* mutation found by MTBDR<sub>sl</sub> testing but not by sequencing, and there was 1 isolate with an *rrs* mutation found by sequencing but not by MTBDR<sub>sl</sub> testing.

Of the 63 *M. tuberculosis* isolates with phenotypic resistance to kanamycin, only 11 were detected either by the presence of an *rrs* mutation (10) or, in one case of detection by the MTBDR<sub>sl</sub> assay, by a lack of binding to *rrs* wild-type probe 1 (ΔWT1). There were 3 isolates with an *rrs* mutation found by the MTBDR<sub>sl</sub> assay but not by sequencing, and there was 1 isolate with an *rrs* mutation found by sequencing but not by the MTBDR<sub>sl</sub> assay. The *rrs* A1401G mutation was not found in any isolates phenotypically suscep-

### TABLE 4 Comparison of MTBDR<sub>sl</sub> testing and analysis of *rrs* and *eis* mutations with phenotypic capreomycin drug susceptibility testing in *Mycobacterium tuberculosis* isolates (n = 112)<sup>a</sup>

<table>
<thead>
<tr>
<th>Capreomycin DST result and no. of isolates</th>
<th>MTBDR&lt;sub&gt;sl&lt;/sub&gt; assay result and mutation(s)</th>
<th>Genetic sequencing result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible (n = 92)</td>
<td>Susceptible</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>C1443G</td>
</tr>
<tr>
<td>10</td>
<td>WT</td>
<td>G-10C</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>G-10A</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>C-14T</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>C159A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Susceptible (n = 5)</td>
<td>Resist</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ΔWT1 + MUT1</td>
<td>A1401G</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1</td>
<td>C-14T</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1</td>
<td>A1401G</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1</td>
<td>C159A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resistant (n = 5)</td>
<td>Resist</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ΔWT1 + MUT1</td>
<td>A1401G</td>
</tr>
<tr>
<td>1</td>
<td>MUT1</td>
<td>A1401G</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1 + MUT1</td>
<td>NA</td>
</tr>
<tr>
<td>Resistential (n = 10)</td>
<td>Susceptible</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>A1401G</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>G-10A</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>C159A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>C-14T</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>DST, drug susceptibility testing; Δ, indicated lack of wild-type band; WT, wild type; NA, not available.

<sup>b</sup>*rrs* gene region or mutation analyzed: WT1, positions 1401 to 1402; MT1, mutation A1401G.

<sup>c</sup>C159A is a silent mutation.
One isolate had a C1443G mutation detected by targeted sequencing which is not associated with kanamycin resistance. Any eis mutation identified was found on targeted sequencing in 33% (21/63) of the isolates with phenotypic resistance compared to 14% (7/49) of those that were phenotypically susceptible ($P < 0.05$). The C-14T mutation was the only eis mutation found solely in kanamycin isolates showing phenotypic resistance.

While the presence of an rrs mutation or any eis mutation had the highest sensitivity (49%) for detecting kanamycin resistance, the specificity was 84%.

### TABLE 5
Comparison of MTBDRsl assay and analysis of rrs and eis mutations with phenotypic kanamycin drug susceptibility testing results in *Mycobacterium tuberculosis* isolates ($n = 112$)

<table>
<thead>
<tr>
<th>Kanamycin DST result and no. of isolates</th>
<th>MTBDRsl assay result</th>
<th>Genetic sequencing result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible ($n = 49$)</td>
<td>Susceptible</td>
<td>rrs</td>
</tr>
<tr>
<td>36</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>C1443G</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>Resistant ($n = 10$)</td>
<td>Resistant</td>
<td>A1401G</td>
</tr>
<tr>
<td>6</td>
<td>ΔWT1 + MUT1</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1 + MUT1</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>ΔWT1 + MUT1</td>
<td>NA</td>
</tr>
<tr>
<td>Resistant ($n = 53$)</td>
<td>Susceptible</td>
<td>A1401G</td>
</tr>
<tr>
<td>8</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>6</td>
<td>WT</td>
<td>WT</td>
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<tr>
<td>4</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>31</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>NA</td>
</tr>
</tbody>
</table>

*DST, drug susceptibility testing; Δ, indicated lack of wild-type band; WT, wild type; NA, not available.*

### TABLE 6
Performance parameters of rrs and eis mutations in detecting any resistance to kanamycin compared to conventional drug susceptibility testing (reference standard)

#### Parameter

<table>
<thead>
<tr>
<th>Value(s)</th>
<th>Capreomycin</th>
<th>Kanamycin</th>
<th>$rrs + eis$</th>
<th>$rrs + C-14T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of isolates showing:</td>
<td>$rrs$ ($n = 112$)</td>
<td>$rrs$ ($n = 112$)</td>
<td>($n = 104$)</td>
<td>($n = 104$)</td>
</tr>
<tr>
<td>True susceptibility</td>
<td>92</td>
<td>49</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>True resistance</td>
<td>6</td>
<td>11</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>False susceptibility</td>
<td>9</td>
<td>52</td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>False resistance</td>
<td>5</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>% sensitivity (95% CI)</td>
<td>40 (15–65)</td>
<td>18 (9–27)</td>
<td>49 (36–62)</td>
<td>27 (16–39)</td>
</tr>
<tr>
<td>% specificity (95% CI)</td>
<td>95 (91–99)</td>
<td>100</td>
<td>84 (74–95)</td>
<td>100</td>
</tr>
<tr>
<td>% PPV (95% CI)</td>
<td>46 (25–84)</td>
<td>100</td>
<td>81 (68–92)</td>
<td>100</td>
</tr>
<tr>
<td>% NPV (95% CI)</td>
<td>91 (86–97)</td>
<td>49 (39–59)</td>
<td>56 (44–68)</td>
<td>51 (41–62)</td>
</tr>
<tr>
<td>Kappa (95% CI)</td>
<td>0.39 (0.14–0.65)</td>
<td>0.16 (0.07–0.25)</td>
<td>0.32 (0.16–0.48)</td>
<td>0.24 (0.13–0.36)</td>
</tr>
</tbody>
</table>

*rrs2 mutations were determined by either MTBDRsl/V1 testing or genetic sequencing. CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.*

*No genetic sequencing results were available for 8 *M. tuberculosis* isolates.*
presence of an \textit{rrs} or \textit{eis} C-14T mutation had lower sensitivity (27%) but retained a high specificity of 100% (Table 6). There were 5 isolates, including isolates from both phenotypically resistant and phenotypically susceptible strains, which showed the presence of a silent C159A mutation.

\textbf{DISCUSSION}

Through the molecular characterization of \textit{M. tuberculosis} isolates from the country of Georgia, we found that detection of mutations in \textit{gyrB} and \textit{eis} genes improved the detection of second-line drug resistance. The inclusion of any mutation in \textit{gyrB} (in addition to mutations in \textit{gyrA}) and the \textit{eis} C-14T mutation (in addition to mutations in \textit{rrs}) increased the sensitivity of detection of phenotypic resistance to ofloxacin and kanamycin by 13% and 9%, respectively. Despite these encouraging findings, however, there was a high rate of \textit{M. tuberculosis} isolates found to be phenotypically resistant to capreomycin and kanamycin without any mutation(s) found in either the \textit{rrs} gene or the \textit{eis} gene. While the inclusion of \textit{gyrB} mutations may increase detection of fluoroquinolone resistance, a better understanding of injectable-drug resistance is still needed to help aid efforts aimed at developing an accurate rapid molecular test.

Among our \textit{M. tuberculosis} isolates, the inclusion of any \textit{gyrB} mutation increased the sensitivity of detection of phenotypic ofloxacin resistance from 75% to 88% and slightly increased the negative predictive value (NPV) for ofloxacin resistance from 96% to 98%. This high NPV would be useful for clinicians in regard to ruling out ofloxacin resistance and in developing an individualized drug resistance treatment regimen. The value of inclusion of \textit{gyrB} mutations to increase the rate of detection of phenotypic fluoroquinolone has been shown to vary in different settings. A study from France found results similar to ours in that the inclusion of \textit{gyrB} mutations increased detection of phenotypic fluoroquinolone resistance by 12% (14), while a large multicenter study performed with \textit{M. tuberculosis} isolates from India, Moldova, Philippines, and South Africa found no benefit of inclusion of \textit{gyrB} mutations (17). Additionally, in a large sequencing study of 1,397 geographically diverse \textit{M. tuberculosis} isolates, the only \textit{gyrB} mutation which was found to have predictive value in analyses of ofloxacin phenotypic resistance was N538T, which was found in only one of our five isolates with a \textit{gyrB} mutation (18).

Among five isolates with \textit{gyrB} mutations and phenotypic ofloxacin resistance, three had additional \textit{gyrA} mutations and two had only a \textit{gyrB} mutation, including R485H and A543V. Results from a functional genetic study suggested that the A543V mutation in isolation was not sufficient to cause fluoroquinolone resistance; however, the mutated Erdman strain used in the study had an elevated ofloxacin MIC of 2 \mu g/ml, and the A543V mutation was found also in two fluoroquinolone-resistant but no fluoroquinolone-susceptible isolates from another study in France (14, 18). Limited data have also not shown an association with R485H phenotypic fluoroquinolone resistance and that phenotypic fluoroquinolone resistance may not be due to a single mutation but may in some cases be due to the interaction of \textit{gyrA} and \textit{gyrB} mutations (19). Thus, in our two cases of phenotypic ofloxacin resistance with only a \textit{gyrB} mutation (R485H, A543V), it is unclear if the resistance was due to these mutations or to an additional, unknown mechanism. If the newer MTBDRslV2 assay were to have been used to detect fluoroquinolone and injectable agent resistance, our two cases of phenotypic ofloxacin resistance with only \textit{gyrB} mutations would not have been detected, as it detects \textit{gyrB} mutations only at codons 536 to 541 (16). Further building of globally diverse \textit{M. tuberculosis} mutation and phenotypic resistance databases such as that by Farhat et al. (19) is necessary to determine the utility of mutations in predicting resistance and which mutations to include in rapid molecular tests.

Although we found \textit{rrs} mutations detected by the MTBDRslV1 assay and/or targeted sequencing to be the most common genetic mutations associated with injectable-drug resistance, their sensitivities for detection of capreomycin resistance (40%) and kanamycin resistance (18%) were extremely low. This low sensitivity has been well documented, especially in Eastern Europe and Russia (10, 12). The addition of any \textit{eis} mutation increased the sensitivity of kanamycin resistance detection from 18% to 49%;
however, the sacrifice was a decrease in specificity from 100% to 84%. In contrast, the inclusion of only the eis C-14T mutation still increased the sensitivity of detection of kanamycin resistance from 18% to 27%, although the increase was less than that seen with the eis mutations, but maintained the high specificity of 100%. Mutations in the eis promoter region were shown to cause aminoglycoside resistance by increasing the amount of aminoglycoside acetyltransferase, thereby leading to drug inactivation (13). There is a preferential effect for resistance to kanamycin over amikacin due to a much higher acetylation rate for kanamycin, and the eis C-14T mutation was associated with the highest kanamycin MIC values compared to G-10A and C-12T mutations. eis mutations are not associated with capreomycin resistance; thus, our finding of similar rates of eis mutations in capreomycin-resistant and -susceptible isolates was not unexpected.

Studies of clinical M. tuberculosis isolates have found that the eis C-14T mutation is very specific for kanamycin resistance whereas the eis C-12T and G-10C mutations are not very specific, being found in many kanamycin-susceptible isolates (12, 15, 16, 20, 21). The eisG-10A mutation has been found to be associated with lower phenotypic resistance in vitro (13) than the C-14T mutation, and while most studies show 100% specificity of the G-10A mutation for phenotypic kanamycin resistance (12, 15), there are a few M. tuberculosis isolates, including two from our study, reported to have the G-10A mutation and susceptibility to kanamycin (20). The emerging data on the differential effects of eis mutations on phenotypic aminoglycoside resistance will be important in guiding clinicians with respect to interpreting the results obtained with the new MTBDRsV2 test, which, in addition to detecting rs mutations, detects mutations in the eis promoter region —10 to —14 (16).

Limitations of our study also included the lack of tlyA sequencing and MIC testing. A prior study from Georgia found no tlyA mutations among 60 M. tuberculosis isolates with phenotypic resistance to capreomycin; thus, tlyA mutations were unlikely to account for our results showing a high proportion of unexplained capreomycin resistance (22). MIC testing would have allowed us to determine if the isolates with an A1401G mutation and capreomycin susceptibility harbored low-level resistance, as has been previously suggested (23). The inclusion of levofloxacin and moxifloxacin DST and MIC testing would have provided important data with respect to clinically utilized fluoroquinolones and the association of certain mutations with low- or high-level resistance as well as fluoroquinolone cross-resistance (24). Additionally, the critical concentration of ofloxacin (2.0 μg/ml) on LJ medium utilized for susceptibility testing was the previously recommended concentration and in 2012 was increased to 4.0 μg/ml. Thus, we may have overestimated ofloxacin phenotypic resistance, particularly in regard to the two M. tuberculosis isolates with phenotypic ofloxacin resistance and no mutations identified. The small number of M. tuberculosis isolates with phenotypic resistance to ofloxacin and capreomycin was also a limitation that led to large confidence intervals for sensitivity calculations. Additionally, as possible reasons for the low sensitivity of genetic mutations for detecting phenotypic capreomycin and kanamycin resistance, we could not rule out errors in DST which may have led to misclassification of M. tuberculosis isolates as falsely resistant or the potential effect of the presence of heteroresistant bacterial populations. Such heteroresistant bacterial populations may have been the cause of the discordance between the MTBDRsV1 assay results and the genetic sequencing results and also of the low sensitivity of genetic mutations in predicting phenotypic resistance, as DST has been shown to be able to detect a lower percentage of resistant bacterial subpopulations (down to 1%) than molecular methods (25, 26). Lastly, we were unable to successfully sequence all our M. tuberculosis isolates, a result which may have been due to the presence of damaged DNA or to deletions or mutations at primer binding sites.

Conclusion. As shown by the results of the study, the inclusion of certain gyrB mutations and eis mutations may increase the sensitivity of detection of ofloxacin and kanamycin resistance, respectively. Continued efforts at building a globally diverse database of M. tuberculosis isolates with detailed genetic and phenotypic DST data will
help clarify which mutations and/or combinations of mutations confer phenotypic drug resistance and which can be feasibly included in a rapid molecular test. The recently released consensus statement from the TBNET and RESIST-TB groups on the clinical implications of molecular drug resistance testing is a great starting point, and we are hopeful that our results, along with those from other studies, will provide the data needed to provide guidance also on \textit{gyrB} and \textit{eis} mutations in the near future \textsuperscript{27}. Additionally, further evaluation on the cause of unexplained kanamycin and capreomycin resistance is needed.

**MATERIALS AND METHODS**

\textbf{Setting.} The study was conducted at the National Reference Laboratory (NRL) of the National Center for Tuberculosis and Lung Diseases (NCTLD) in Tbilisi, Georgia, where all cultures and drug susceptibility testing were performed. The NRL has undergone external quality assessment by the Antwerp WHO Supranational TB Reference Laboratory each year since 2005. Targeted DNA sequencing was performed at the Public Health Research Institute (PHRI) TB Center in Newark, NJ, USA.

\textbf{Study samples, culture, and DST.} The \textit{M. tuberculosis} isolates used for this study were obtained from a frozen collection and were mostly from patients who had been enrolled in a previous study evaluating the performance of the MTBDR\textit{sl} assay \textsuperscript{11}. The study population consisted of consecutive patients with documented MDR TB from throughout the country of Georgia during the period of November 2011 to April 2012. A total of 112 \textit{M. tuberculosis} isolates from the 143 culture-positive patients with complete MTBDR\textit{sl} V1 results in our prior study were available and able to be subcultured at the NRL per standard methodology \textsuperscript{7}. After frozen isolates were thawed at room temperature, a 0.5-ml suspension was inoculated onto two Lowenstein-Jensen (LJ) slants, which were incubated at 37°C until confluent growth was observed. DST of second-line drugs was previously done on all the isolates using the proportion method and LJ medium with the following drug concentrations: for ofloxacin, 2.0 \(\mu\)g/ml; for para-aminosalicylic acid, 0.5 \(\mu\)g/ml; for capreomycin, 40.0 \(\mu\)g/ml; for kanamycin, 30.0 \(\mu\)g/ml \textsuperscript{11}.

\textbf{DNA extraction and sequencing.} DNA extraction was performed using a QIAamp DNA minikit (Qiagen Inc., Valencia, CA). DNA extracts were shipped to the NCTLD to the PHRI, where targeted genetic sequencing was performed. Genomic DNA was subjected to PCR (the primers used for the \textit{gyrA}, \textit{gyrB}, and \textit{rrs} genes and for the \textit{eis} gene and its promoter are listed in Table 1) followed by Sanger sequencing of coding DNA sequences. The positions corresponding to the beginning and end for each amplicon were based on the map of the \textit{M. tuberculosis} H37\textit{Rv} reference strain using Tuberculist (http://genolist.pasteur.fr/Tuberculist/) and are as follows: for \textit{gyrA}, positions 7340 to 7699; for \textit{gyrB}, 6968 to 7064; for \textit{rrs}, 1472694 to 1473446; for the \textit{eis} gene, 2714035 to 2714923; for the \textit{eis} promoter, 2714594 to 2715411. Mutations were identified by alignment of nucleotide sequences to those of the \textit{M. tuberculosis} H37\textit{Rv} reference strain (NCBI accession number AL123456) \textsuperscript{28} using ClustalW2 as previously described \textsuperscript{17}. The MTBDR\textit{sl} V1 results from our prior study were available, and the test was performed as previously described \textsuperscript{11}.

\textbf{Data analysis.} All data analyses were carried out using SAS, version 9.4 (Statistical Analysis Software Institute, Cary, NC, USA). The sensitivity, specificity, positive predictive value, and negative predictive value corresponding to mutations in certain genes detecting phenotypic resistance to ofloxacin (\textit{gyrA}, \textit{gyrB}), capreomycin (\textit{rrs}, \textit{eis} promoter), and kanamycin (\textit{rrs}, \textit{eis} promoter) were calculated using conventional DST results as the reference standard. For these calculations, if a mutation in the \textit{gyrA} or \textit{rrs} gene was found either by the MTBDR\textit{sl} V1 assay or by genetic sequencing, it was considered present. No mutations were found in the \textit{eis} gene; thus, only \textit{eis} promoter mutations were included in the data analysis.

\textbf{Accession number(s).} Data have been deposited in GenBank under accession no. MF098543.

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We declare that we have no conflicts of interest.

**REFERENCES**


