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MAJOR ARTICLE

Resistance-Conferring Mutations on Whole-Genome Sequencing of Fluoroquinolone-resistant and -Susceptible *Mycobacterium tuberculosis* Isolates: A Proposed Threshold for Identifying Resistance

Fernanda Maruri,[1,2] Yan Guo,[3] Amondrea Blackman,[1,2] Yuri F. van der Heijden,[1,2,4] Peter F. Rebeiro,[1,2] and Timothy R. Sterling[1,2]

[1]Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA, [2]Vanderbilt Tuberculosis Center, Vanderbilt University School of Medicine, Nashville, Tennessee, USA, [3]Department of Internal Medicine, University of New Mexico, Albuquerque, New Mexico, USA, and [4]The Aurum Institute, Johannesburg, South Africa

Background. Fluoroquinolone resistance in *Mycobacterium tuberculosis* (Mtb) is conferred by DNA gyrase mutations, but not all fluoroquinolone-resistant Mtb isolates have mutations detected. The optimal allele frequency threshold to identify resistance-conferring mutations by whole-genome sequencing is unknown.

Methods. Phenotypically ofloxacin-resistant and lineage-matched ofloxacin-susceptible Mtb isolates underwent whole-genome sequencing at an average coverage depth of 868 reads. Polymorphisms within the quinolone-resistance–determining region (QRDR) of *gyrA* and *gyrB* were identified. The allele frequency threshold using the Genome Analysis Toolkit pipeline was ~8%; allele-level data identified the predominant variant allele frequency and mutational burden (ie, sum of all variant allele frequencies in the QRDR) in *gyrA*, *gyrB*, and *gyrA* + *gyrB* for each isolate. Receiver operating characteristic (ROC) curves assessed the optimal measure of allele frequency and potential thresholds for identifying phenotypically resistant isolates.

Results. Of 42 ofloxacin-resistant Mtb isolates, area under the ROC curve (AUC) was highest for predominant variant allele frequency, so that measure was used to evaluate optimal mutation detection thresholds. AUCs for 8%, 2.5%, and 0.8% thresholds were 0.8452, 0.9286, and 0.9069, respectively. Sensitivity and specificity were 69% and 100% for 8%, 86% and 100% for 2.5%, 91% and 91% for 0.8%. The sensitivity of the 2.5% and 0.8% thresholds were significantly higher than the 8% threshold ($P = .016$ and .004, respectively) but not significantly different between one another ($P = .5$).

Conclusions. A predominant mutation allele frequency threshold of 2.5% had the highest AUC for detecting DNA gyrase mutations that confer ofloxacin resistance, and was therefore the optimal threshold.

Keywords. *M. tuberculosis*; fluoroquinolone resistance; whole-genome sequencing.

Fluoroquinolones have potent bactericidal activity against *Mycobacterium tuberculosis*; they act by binding to DNA gyrase and preventing negative supercoiling of replicating DNA. Mutations in *gyrA* and *gyrB*, the genes that encode the 2 subunits of DNA gyrase, are the most frequent mechanism of fluoroquinolone resistance [1, 2]. Such mutations confer resistance by interfering with fluoroquinolones binding to *M. tuberculosis* in the quinolone binding pocket (QBP), within the quinolone-resistance–determining region (QRDR). The QRDR is composed of codons 74–113 in *gyrA* and 461–499 in *gyrB* (using the P0C5C5|1–675 numbering scheme) [3]. Recent studies have identified fluoroquinolone-resistance–conferring mutations immediately outside the traditional QRDR of *gyrB*, at codons 500 and 501. These 2 codons are in the QBP and in direct contact with the quinolone molecule. These studies refined the definition of the QRDR in *gyrB*, extending it from position 461 to 501 [1, 4].

Whole-genome sequencing is a sensitive technique for identifying resistance mutations. However, not all fluoroquinolone-resistant *M. tuberculosis* isolates have mutations identified in DNA gyrase [2, 5–7]. Although web-based tools have been developed to improve resistance-conferring mutation identification, a consensus regarding the depth of coverage required for whole-genome sequencing to detect drug resistance in *M. tuberculosis* has not been established [6, 8].

In a previous study, we noted heteroresistance in 10 of 26 (38%) *M. tuberculosis* isolates phenotypically resistant to ofloxacin. Allele frequencies ranging from 4% to 84% were observed in the heteroresistant isolates [9].

Although diagnostic tests accurately detect resistance to first-line antituberculosis (-TB) drugs such as isoniazid and rifampin, more sensitive tests are needed to detect fluoroquinolone

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Correspondence: F. Maruri, A2209 Medical Center North, 1161 21st Avenue South, Nashville, TN 37232 (fernanda.maruri@vumc.org).

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resistance [10, 11]. A recent study using targeted next-generation sequencing found that microheteroresistance exists below the resolving capability of conventional genotypic tests [12].

In this study, we sought to extend our previous findings by reporting on the allele-level frequency of variants seen in fluoroquinolone-susceptible and -resistant *M. tuberculosis* isolates. We investigated whether a widely used genome-wide bioinformatics pipeline missed low-level fluoroquinolone-resistant variants (ie, microheteroresistance), and propose using allele-level analysis at an allele frequency threshold that optimizes sensitivity and specificity of genotypic fluoroquinolone resistance, to complement whole-genome analysis.

METHODS

Study Overview

We performed whole-genome sequencing and assessed allele-level data for DNA gyrase variants in 3 groups of *M. tuberculosis* isolates: (1) phenotypically ofloxacin-resistant isolates with *gyrA/B* mutations identified on whole-genome sequencing, (2) ofloxacin-resistant isolates without *gyrA/B* mutations identified on whole-genome sequencing, and (3) ofloxacin-susceptible isolates.

Study Population

Ofloxacin-resistant *M. tuberculosis* isolates were identified in a population-based study of patients with newly diagnosed, culture-confirmed TB reported in Tennessee from 2002 to 2016. Eleven phenotypically ofloxacin-susceptible isolates were selected on the basis of genotypic lineage, which was determined by spoligotype and 12-locus mycobacterial interspersed repetitive-unit (MIRU) compared with 13 ofloxacin-resistant isolates that did not have *gyrA/B* mutations by whole-genome sequencing [13]. Controls for these latter isolates were felt to be most important, since they were felt likely to have an alternative fluoroquinolone-resistance mechanism. One fluoroquinolone-resistant isolate of Indo-Oceanic lineage did not have any matched fluoroquinolone-susceptible isolates.

Resistance Testing

All isolates in this study were tested at Vanderbilt University Medical Center (VUMC) by 2 different drug-susceptibility methods: agar proportion and the resazurin microtiter assay (REMA). Briefly, a suspension with a turbidity equivalent to a 1.0 McFarland standard was prepared from <14-day-old *M. tuberculosis* colonies grown on Lowenstein–Jensen medium. After confirmation of turbidity by nephelometer, the suspension served as the standard inoculum for all dilutions. One hundred microliters each of 10^{-2} and 10^{-4} dilutions of the standard inoculum were plated on 7H10 agar with and without ofloxacin. Phenotypic resistance was defined as more than 1% colony growth in the presence of 2 mg/L ofloxacin compared with colony growth in the absence of drug.

REMA has been validated for ofloxacin resistance testing in *M. tuberculosis*, using a critical concentration of 2 mg/L [9, 14, 15]. The ofloxacin (Sigma-Aldrich, St Louis, MO) minimum inhibitory concentration (MIC) was assessed at 2-fold dilutions between 0.5 and 64 mg/L. If growth was observed at day 7 in the control well, 30 µL of 0.01% resazurin solution was added to each well, followed by 24 hours of incubation. Color change indicated bacterial growth and MIC was determined as the lowest ofloxacin concentration preventing color change. The critical concentration of ofloxacin was 2 mg/L. The MIC reported and used for analyses was the median of 3 tests performed (Supplementary Table 1).

Molecular Typing

Spoligotyping and 12-locus MIRU typing were performed by the Michigan Department of Community Health on all *M. tuberculosis* isolates from culture-confirmed TB cases in Tennessee from 2004 to 2016 and a select number of isolates from 2002 and 2003 [13].

Whole-Genome Sequencing

Each isolate was grown on Lowenstein-Jensen media in the absence of ofloxacin. Multiple colonies from each isolate were emulsified in 200 µL nuclease-free water. Genomic DNA was isolated from the cell suspension and purified using the ZR Bacterial/Fungal DNA Mini-prep Kit (Zymo Research, Irvine, CA). Samples were then heated to 100°C for 10 minutes. The DNA concentration was verified by NanoDrop (Thermo Scientific, Waltham, MA).

Whole-genome sequencing was performed at the Genome Sciences Resource at VUMC for 53 *M. tuberculosis* clinical isolates (42 phenotypically ofloxacin resistant, 11 susceptible). The Illumina HiSEq, HiSEq 3000, and Novaseq platforms generated paired-end reads of 100 and 150 bp with an average depth of coverage of 868 reads (Supplementary Table 2). Raw base calls were generated from the internal Casava pipeline software (Illumina, San Diego, CA). Output was in the fastq format. Sequences were aligned and mapped to the reference H37Rv genome (Genbank Al123456.3) using the Burrows Wheeler Aligner [16]. The alignments were sorted and indexed using SAMtools [17]. The unified genotyper from the Genome Analysis Toolkit (GATK) was then used to make variant calls [18]. GATK output contained an unfiltered list of single nucleotide polymorphisms (SNPs) in the variant call format. SNPeff was used to annotate output base changes and determine whether mutations were synonymous [19].

Allele-level Analysis

For each sample, allele frequencies of the 4 nucleotides at 27 selected loci in *gyrA* and *gyrB* were computed from pileup results of Binary Alignment Map (BAM) files, by counting reads aligned to each nucleotide. Sequencing data went through

rigorous quality-control procedures. Sequence reads with a mapping quality Phred score less than 20 (reads with error rate >1%) were removed from the analysis. Low-quality reads were filtered out before allele frequencies were counted [20].

Genomic positions were selected from codons in the QRDRs of *gyrA/B* that were confirmed to confer fluoroquinolone resistance via functional analysis; this was determined by review of the literature and available databases (ReSeqTB) [2, 4–7, 21–33] (Supplementary Table 3).

Statistical Methods

The predominant DNA gyrase mutation was defined as the variant with the highest nucleotide allele frequency among the selected loci in *gyrA/B*. The mutational burden in *gyrA* was determined by summing all variant allele frequencies in the *gyrA* loci. The same process was performed among the selected loci in *gyrB* to determine the mutational burden in *gyrB*. The total mutational burden was the sum of variant allele frequencies in *gyrA/B*.

The Kruskal-Wallis and Wilcoxon rank-sum tests assessed differences in the distribution of predominant allele frequency and mutational burdens among resistant and susceptible isolates.

To identify the measure of variant allele frequency that correlated most closely with phenotypic fluoroquinolone resistance, we performed logistic regression of the binary phenotypic resistance outcome on the continuous allele frequency exposure to generate a receiver operating characteristic (ROC) curve and calculated the area under the curve (AUC). These analyses are used to assess the discrimination of a binary outcome (eg, resistant vs not resistant) using predictors (eg, allele frequency), and to find the optimal sensitivity and specificity threshold values of a test and compare these values across more than 1 diagnostic test [34, 35]. The ROC of a test with perfect discrimination passes through the left-upper corner of the unit square (the point where the sensitivity and specificity are equal to 1) [36]. Each observation generates a binary response classification matrix in the form of a predicted probability of the positive or negative result. A threshold on the predictive probability scale can be selected, above which the test is positive. Considering phenotypic drug-susceptibility testing as the reference standard, we compared it with (1) the mutation in *gyrA* or *gyrB* with the predominant allele frequency, (2) the mutational burden in *gyrA*, (3) the mutational burden in *gyrB*, and (4) the total mutational burden.

Subsequently, to identify the optimal allele frequency cutoff (genotypic threshold) for detecting phenotypic fluoroquinolone resistance, we used 3 commonly used optimality criteria for determining threshold values: the point on the ROC curve (1) with minimum distance from the left-upper corner of the unit square, (2) where the sensitivity and specificity of the test were equal, and (3) where the sensitivity and specificity were maximized (Youden's index). These 3 criteria weight sensitivity and specificity equally [36].

Once the optimal threshold values were determined, we assessed the allele frequency detection threshold of whole-genome sequencing analysis and considered the lowest allele frequency that made a variant call in the genome-wide analysis as the threshold. We calculated the sensitivity and specificity when using each of the optimal thresholds and compared them with the genome-wide analysis threshold using the McNemar's test to determine statistical significance. SAS system for Windows version 7.15 HF7 (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

There were 42 ofloxacin-resistant *M. tuberculosis* isolates during the study period, of which 29 (69%) had resistance-conferring mutations in *gyrA* or *gyrB* on whole-genome sequencing and 13 (31%) did not. There were 11 ofloxacin-susceptible *M. tuberculosis* isolates. Output files were combined for all 53 isolates. After removing SNPs found in susceptible isolates and synonymous SNPs, 19 unique nonsynonymous SNPs were identified in *gyrA* or *gyrB* on whole-genome sequencing; 11 of 19 were inside the QRDR of *gyrA* or *gyrB*.

Table 1 shows all nonsynonymous mutations in the QRDRs of *gyrA/B* found in the study isolates. Five of the mutations have previously been shown to confer resistance by functional analysis; the remaining mutations have been reported in fluoroquinolone-resistant isolates [2, 25]. Mutations at positions 7582(D94G) and 7581(D94N) were the most frequent mutations in *gyrA*, found in 13 and 7 ofloxacin-resistant isolates, respectively. The A90V mutation at position 7570 was seen in 6 ofloxacin-resistant isolates and the mutations at positions

Table 1. All Nonsynonymous Mutations at Codons in DNA Gyrase That Have Been Associated With Fluoroquinolone Resistance in *Mycobacterium tuberculosis* Identified in Our Study Population of *M. tuberculosis* Isolates

Name	Gene	Position	AA_Change	Codon Change[a]
gyrA	Rv0006	7566	D89N	Gac/Aac
gyrA	Rv0006	7570	A90V[b]	gCg/gTg
gyrA	Rv0006	7572	S91P	Tcg/Ccg
gyrA	Rv0006	7581	D94N	Gac/Aac
gyrA	Rv0006	7581	D94Y	Gac/Tac
gyrA	Rv0006	7582	D94A	gAc/gCc
gyrA	Rv0006	7582	D94G[b]	gAc/gGc
gyrB	Rv0005	6620	D461N[b]	Gac/Aac
gyrB	Rv0005	6620	D461H[b]	Gac/Cac
gyrB	Rv0005	6735	N499I	aAc/aTc
gyrB	Rv0005	6738	T500N[b]	aCc/aAc

The codon, not necessarily the mutation, has been associated with fluoroquinolone resistance.

Abbreviation: AA, amino acid.

[a]Capital letters denote nucleotide change.

[b]Fluoroquinolone resistance mutations previously shown to confer resistance by functional analysis.

7572(S91P) and 7581(D94Y) were each seen in 3 ofloxacin-resistant isolates. The D94A and D89N at positions 7582 and 7566 were each seen in 1 ofloxacin-resistant isolate.

Four mutations were found in *gyrB* at codons 461(D461N and D461H), 499(N499I), and 500(T500N), each appearing in 1 ofloxacin-resistant isolate. Mutations at codons 461(D461H) and 500 were seen in isolates that also had *gyrA* mutations.

Of the 11 ofloxacin-susceptible isolates, none had resistance-conferring mutations in the QRDRs of *gyrA/B*.

Allele-level Analysis

Allele frequencies were obtained for 27 selected genomic positions (15 in *gyrA* and 12 in *gyrB*); 23 genomic positions remained after removing the positions resulting in synonymous substitutions (12 in *gyrA* and 11 in *gyrB*). Table 2 shows the allele frequency of the predominant DNA gyrase mutation and the *gyrA*, *gyrB*, and total mutational burdens for the 29 fluoroquinolone-resistant isolates with DNA gyrase mutations identified in the genome-wide analysis. The lowest allele frequency seen was 8%. Nucleotides with lower allele frequencies were not called variants in the genome-wide analysis. Table 3 shows the predominant allele frequency and the *gyrA*, *gyrB*, and total mutational burdens for the 13 fluoroquinolone-resistant isolates without DNA gyrase mutations by whole-genome sequencing analysis. The highest allele frequency was 6.8% and the lowest was 0.2%; 1 isolate had no allele frequencies greater than 0. Supplementary Table 4 includes all fluoroquinolone-resistant isolates from Tables 2 and 3, listed according to predominant allele frequency.

In Table 4 the highest predominant allele frequency of the 11 fluoroquinolone-susceptible isolates was 1.7% and the lowest was 0.1%. We compared variant allele frequencies between the 3 groups of isolates. The differences in median allele frequencies between fluoroquinolone-resistant isolates with DNA gyrase mutations, without DNA gyrase mutations, and fluoroquinolone-susceptible isolates were all statistically significant for the predominant allele frequency, the mutational burden in *gyrA*, and the total mutational burden. However, the differences were not statistically significant for the *gyrB* mutational burden (Table 5).

Receiver Operating Characteristic Analyses

Figure 1 shows the ROC curve comparison to determine which measure (ie, the predominant allele frequency, *gyrA* mutational

Table 2. DNA Gyrase Allele Frequencies of Fluoroquinolone-resistant *Mycobacterium tuberculosis* Isolates With Mutations Identified on Whole-Genome Sequencing at Codons Known to Confer Fluoroquinolone Resistance

Study ID	MIC	Predominant DNA Gyrase Mutation Allele Frequency	Mutational Burden[a]			Predominant DNA Gyrase Mutation
			gyrA	*gyrB*	*gyrA* Plus *gyrB*	
1	8	1.0000	1.0110	0.0059	1.0169	D94G
2	8	1.0000	1.0097	0.0027	1.0125	D94Y
3	8	1.0000	1.0082	0.0097	1.0180	D94G
4	16	0.9992	1.0177	0.0117	1.0295	D94G
5	32	0.9989	1.0157	0.0156	1.0313	D94G
6	16	0.9988	1.0178	0.0123	1.0301	S91P
7	4	0.9985	1.0217	0.0159	1.0376	A90V
8	16	0.9980	1.0095	0.0057	1.0151	D94G
9	16	0.9976	1.0110	0.0067	1.0177	D94Y
10	16	0.9976	1.0085	0.0123	1.0209	D94N
11	16	0.9975	0.9987	0.0033	1.0020	D94N
12	16	0.9960	1.0156	0.0096	1.0252	D94G
13	16	0.9772	1.0185	0.0082	1.0268	D94N
14	16	0.9686	0.9988	0.0091	1.0079	D94N
15	16	0.9621	0.9918	0.0260	1.0179	D94G
16	4	0.9583	0.0000	0.9583	0.9583	N499I
17	8	0.8098	0.8210	0.2067	1.0276	A90V
18	8	0.8060	1.0059	0.0133	1.0192	D94N
19	16	0.7983	0.9707	0.0035	0.9742	D94Y
20	16	0.6322	1.0161	0.0068	1.0230	A90V
21	8	0.4946	0.9958	0.0106	1.0064	D94N
22	16	0.4775	0.4841	0.0015	0.4856	D94G
23	16	0.4393	0.7420	0.2193	0.9613	D94G
24	8	0.3491	0.3491	0.0013	0.3504	D94G
25	4	0.2611	0.4706	0.0013	0.4719	D94N
26	8	0.2256	0.4609	0.0050	0.4659	S91P
27	4	0.0987	0.0110	0.1044	0.1153	D461N
28	4	0.0890	0.0930	0.0038	0.0968	D94A
29	4	0.0802[b]	0.0938	0.0000	0.0938	A90V

N = 29.

Abbreviation: MIC, minimum inhibitory concentration.

[a]Mutational burden: sum of mutation allele frequencies at resistance-conferring codons in the quinolone-resistance determining region (QRDR) of *gyrA, gyrB*, or both combined.

[b]Mutation detection threshold by genome-wide analysis.

Table 3. DNA Gyrase Allele Frequencies of Fluoroquinolone-resistant *Mycobacterium tuberculosis* Isolates Without Mutations Identified on Whole-Genome Sequencing at Codons Known to Confer Fluoroquinolone Resistance

Study ID	MIC	Predominant DNA Gyrase Mutation Allele Frequency	Mutational Burden[a]			Predominant DNA Gyrase Mutation
			gyrA	*gyrB*	*gyrA* Plus *gyrB*	
30	4	0.0675	0.0734	0.0018	0.0752	D94G
31	8	0.0594	0.0741	0.0086	0.0826	D94H
32	8	0.0481	0.0520	0.0010	0.0531	D94N
33	8	0.0290	0.0386	0.0000	0.0386	D94Y
34	2	0.0273	0.0371	0.0092	0.0463	D94G
35	8	0.0261	0.0348	0.0080	0.0429	D94G
36	8	0.0253	0.0323	0.0053	0.0376	D94N
37	8	0.0092	0.0223	0.0142	0.0365	D89Y
38	4	0.0075	0.0052	0.0075	0.0127	D461Y
39	8	0.0054	0.0143	0.0051	0.0193	D94N
40	8	0.0041	0.0164	0.0041	0.0205	D461Y
41	16	0.0021	0.0077	0.0038	0.0115	A90T
42[b]	4	0.0000	0.0000	0.0000	0.0000	…

N = 13.

Abbreviation: MIC, minimum inhibitory concentration.

[a]Mutational burden: sum of variant allele frequencies at resistance-conferring codons in the quinolone-resistance determining region (QRDR) of *gyrA, gyrB*, or both combined.

[b]In this isolate, all allele frequencies at the selected positions were 0.

Table 4. DNA Gyrase Allele Frequencies of Fluoroquinolone-Susceptible *Mycobacterium tuberculosis* Isolates

Study ID	MIC	Predominant DNA Gyrase Mutation Allele Frequency	Mutational Burden[a]			Predominant DNA Gyrase Mutation
			gyrA	*gyrB*	*gyrA* Plus *gyrB*	
43	1	0.0173	0.0478	0.0142	0.0619	D89Y
44	1	0.0063	0.0255	0.0056	0.0311	D94Y
45	1	0.0060	0.0201	0.0122	0.0323	D94Y
46	1	0.0028	0.0122	0.0060	0.0182	D461Y
47	1	0.0025	0.0038	0.0049	0.0087	G88S
48	1	0.0025	0.0046	0.0025	0.0071	D461Y
49	1	0.0019	0.0019	0.0024	0.0043	S91W
50	1	0.0018	0.0020	0.0026	0.0047	D461H
51	1	0.0016	0.0025	0.0030	0.0054	D94N
52	1	0.0015	0.0043	0.0025	0.0068	A90V
53	1	0.0013	0.0026	0.0033	0.0059	A90G

None of these isolates had mutations identified on whole-genome sequencing at codons known to be associated with fluoroquinolone resistance (N = 11).

Abbreviation: MIC, minimum inhibitory concentration.

[a]Mutational burden: sum of variant allele frequencies at resistance-conferring codons in the quinolone-resistance determining region (QRDR) of *gyrA*, *gyrB*, or both combined.

burden, *gyrB* mutational burden, or total DNA gyrase mutational burden) had the best predictive accuracy, using phenotypic drug susceptibility testing (DST) as the reference standard. The AUC of the predominant mutation allele frequency was highest at 0.95, and the *gyrB* mutational burden was the lowest at 0.64. The confidence interval for *gyrB* mutational burden (Figure 1) contains .5; hence, it is not significantly different from a random guess, which is represented by the diagonal line. The AUC is used as an index of discriminating ability; therefore, the measure with the highest AUC (ie, predominant mutation allele frequency) was used as the measure to predict fluoroquinolone resistance.

To analyze the effectiveness of the predominant mutation allele frequency as a predictor of fluoroquinolone resistance, we performed a second ROC curve analysis to identify optimal thresholds on the ROC curve using the 3 optimality criteria described above. Figure 2 shows the ROC curve with the optimal thresholds identified corresponding to the predominant allele frequency of 0.025 (2.5%) by 1 of the criteria used: Youden's index. The second and third criteria—the minimum distance from the left-upper corner to the unit square and the point on the ROC curve where the sensitivity and specificity are equal—show a threshold corresponding to a predominant allele frequency of 0.008 (0.8%). The sensitivity and specificity of the 0.008 threshold was 91%, while the sensitivity and specificity of the 0.025 threshold were 86% and 100%, respectively (Figure 2, Table 6, Supplementary Tables 5 and 6).

We performed a third ROC curve analysis (Figure 3) to determine which threshold had the highest AUC. We compared the predominant mutation allele frequency threshold observed in the genome-wide analysis (8%) with the 2 predominant mutation allele frequency thresholds determined by the threshold optimality analysis (2.5% and 0.8%). The predominant mutation allele frequency of 2.5% had the highest AUC at 0.93, significantly higher than the 8% threshold (AUC = 0.85; $P = .042$). The differences in AUC between the 8% and 0.8% predominant mutation allele frequencies and between the 2.5% and 0.8% were not significantly different. These results were confirmed by the McNemar's test of sensitivities used to compare 2 diagnostic tests. The sensitivities of the diagnostic tests using the 8% and 2.5% predominant allele frequency thresholds were significantly different ($P = .016$) (Table 7). The McNemar's test of sensitivities also showed a statistically significant difference between the sensitivities of the 8% and 0.8% thresholds ($P = .004$) (Table 7), but not between the 2.5% and 0.8% thresholds ($P = .5$).

DISCUSSION

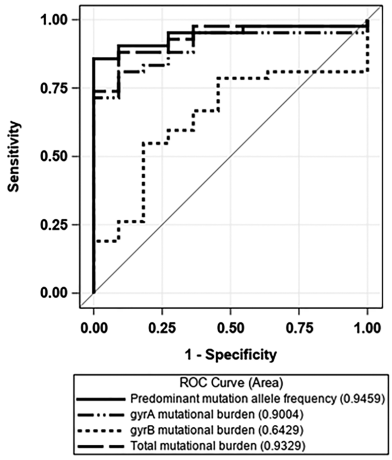
There were several important findings of this study. First, the proportion of fluoroquinolone-resistant isolates without DNA

Table 5. Differences in Median Mutation/Variant Allele Frequencies Among Fluoroquinolone-resistant *Mycobacterium tuberculosis* Isolates With and Without DNA Gyrase Mutations on Whole-Genome Sequencing and Fluoroquinolone-Susceptible Isolates

Isolate Group[a]	N	Predominant DNA Gyrase Mutation/ Variant Allele Frequency (Median)	*P*	Mutational Burden *gyrA* (Median)	*P*	Mutational Burden *gyrB* (Median)	*P*	Mutational Burden *gyrA* Plus *gyrB* (Median)	*P*
FQR with *gyr* mutations	29	0.9621	<.0001	0.9988	<.0001	0.0091	.0519	1.0151	<.0001
FQR without *gyr* mutations	13	0.0253		0.0323		0.0051		0.0376	
FQS	11	0.0025		0.0043		0.0033		0.0071	
FQR with *gyr* mutations	29	0.9621	<.0001	0.9988	<.0001	0.0091	.0517	1.0151	<.0001
FQR without *gyr* mutations	13	0.0253		0.0323		0.0051		0.0376	
FQR with *gyr* mutations	29	0.9621	<.0001	0.9988	<.0001	0.0091	.0579	1.0151	<.0001
FQS	11	0.0025		0.0043		0.0033		0.0071	
FQR without *gyr* mutations	13	0.0253	.0130	0.0323	.0279	0.0051	.9315	0.0376	.0279
FQS	11	0.0025		0.0043		0.0033		0.0071	

Abbreviations: FQR, fluoroquinolone-resistant; FQS, fluoroquinolone-susceptible.

[a]Comparisons using 3 levels done by Kruskal-Wallis test; pairwise comparisons done by Wilcoxon rank-sum test.



ROC Association Statistics

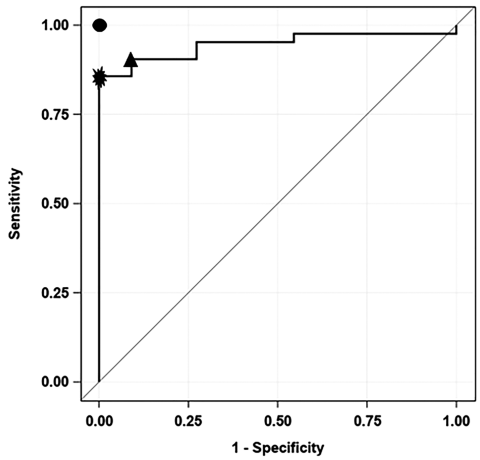
ROC Model	Mann-Whitney			
	Area	Standard Error	95% Wald Confidence Limits	
Predominant mutation allele frequency	0.9459	0.0304	0.8863	1.0000
gyrA mutational burden	0.9004	0.0434	0.8154	0.9854
gyrB mutational burden	0.6429	0.0872	0.4720	0.8137
Total mutational burden	0.9329	0.0354	0.8635	1.0000

Figure 1. ROC plot comparing phenotypic DST result with the proportion of reads associated with fluoroquinolone resistance in *Mycobacterium tuberculosis* DNA gyrase: (1) the mutation in *gyrA* or *gyrB* with the predominant allele frequency, (2) the sum of variant allele frequencies in *gyrA (gyrA* mutational burden), (3) the sum of variant allele frequencies in *gyrB (gyrB* mutational burden), or (4) the sum of variant allele frequencies in *gyrA* plus *gyrB* (total mutational burden). Abbreviations: DST, drug susceptibility test; ROC, receiver operating characteristic.

gyrase mutations identified on whole-genome sequencing (31%) was high and consistent with previous reports [2]. These findings support recent studies demonstrating the need for more sensitive genotypic resistance tests to detect fluoroquinolone resistance in *M. tuberculosis* [10, 11, 37–39]. Second, widely available pipelines miss phenotypic fluoroquinolone resistance in isolates that have low-level allele frequencies (microheteroresistance); therefore, allele frequency analysis after undergoing rigorous quality-control procedures could be used in conjunction with whole-genome sequencing to improve sensitivity. Third, the predominant DNA gyrase mutation allele frequency provided greater discriminating capacity than the mutational burden in *gyrA*, *gyrB*, or the combination of *gyrA* plus *gyrB*. Fourth, an allele frequency mutation detection threshold of 2.5% provided significantly greater sensitivity for detecting resistance than whole-genome sequencing analysis done with a widely available pipeline; sensitivity increased from 69% to 86% without sacrificing specificity (Table 6). This approach provides a more sensitive way of detecting fluoroquinolone resistance, especially on heteroresistant strains that are missed by the detection limits of many phenotypic and molecular assays [40]. It should be noted that this occurred with an average depth of coverage of 868 reads.

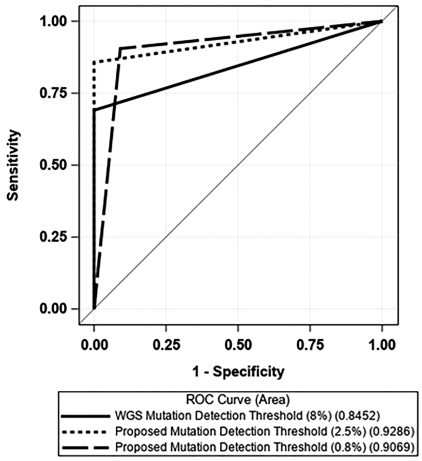
Importantly, even with more sensitive diagnostic tests, in this cohort of 42 ofloxacin-resistant *M. tuberculosis* isolates, 6 (14%) would still not be detected by genotypic methods using the 2.5% threshold and 4 (10%) isolates would not have been detected by using 0.8%. This suggests that mechanisms of fluoroquinolone resistance in *M. tuberculosis* other than those mediated by DNA gyrase mutations may be involved. If alternative resistance mechanisms and associated mutations are identified, they could potentially be included in diagnostic tests to further increase sensitivity.

The predominance of *gyrA* D94G, D94N, A90V, and S91P mutations in ofloxacin-resistant isolates is consistent with previously published studies [5].



Criterion name	Threshold applied to predictive probability	Predominant mutation allele frequency	Sensitivity	Specificity	Criterion value
Distance to 0,1	0.47279	0.008	0.905	0.909	0.13166[a]
Sensitivity-Specificity equality	0.47279	0.008	0.905	0.909	0.00433[b]
Youden's index	0.91371	0.025	0.857	1.000	0.85714[c]

Figure 2. ROC plot comparing phenotypic DST with the predominant mutation allele frequency in *gyrA* or *gyrB* associated with fluoroquinolone resistance in *Mycobacterium tuberculosis* to identify the optimal genotypic threshold for detecting phenotypic resistance. A threshold of >2.5% was associated with the best sensitivity and specificity for detecting phenotypic fluoroquinolone resistance. The point at the left-upper corner shows where sensitivity = 1 and specificity = 1 or 100% (perfect test) is denoted by the filled circle. The optimal threshold corresponding to 0.025 predominant mutation allele frequency (denoted by the filled star) identified by Youden's index criterion (the point on the ROC curve where sensitivity + specificity − 1 is maximum). The optimal threshold corresponding to 0.008 predominant allele frequency (denoted by the filled triangle) identified by distance to 0, 1 criterion (the point on the ROC curve where the distance to the perfect test is minimum). [a]Criterion value derived from the distance to the perfect point in the unit square [√ ([1 – sensitivity] [2] + [1 – specificity] [2]) = 0.131]. [b]Criterion value derived from the difference between sensitivity and specificity (sensitivity − specificity = 0.004). [c]Criterion value derived from the Youden's index (sensitivity + specificity – 1 = 0.857). Abbreviations: DST, drug susceptibility test; ROC, receiver operating characteristic.



Contrast	Standard Error	95% Wald Confidence Limits		Chi-Square	Pr > ChiSq
WGS Mutation Detection Threshold (8%) - Proposed Mutation Detection Threshold (2.5%)	0.0291	-0.1404	-0.0263	8.2000	0.0042
WGS Mutation Detection Threshold (8%) - Proposed Mutation Detection Threshold (0.8%)	0.0556	-0.1707	0.0473	1.2304	0.2673
Proposed Mutation Detection Threshold (2.5%) - Proposed Mutation Detection Threshold (0.8%)	0.0484	-0.0732	0.1165	0.2000	0.6547

Figure 3. Comparison of mutation allele frequency thresholds for fluoroquinolone resistance. Abbreviations: ROC, receiver operating characteristic; WGS, whole-genome sequencing.

There were several limitations of this study. First, this relatively small sample of ofloxacin-resistant isolates in Tennessee was of predominantly European-American lineage, with few East-Asian strains. The study may have been underpowered to detect novel resistance-conferring mutations since genetic background has been reported to affect both ofloxacin MIC and ofloxacin resistance mutational profiles [41] and could have affected the optimal predominant mutation allele frequency for detecting fluoroquinolone resistance. It is unclear if these results are generalizable to other settings. Second, the list of mutations associated with fluoroquinolone resistance, and the selected genomic positions used in our allele-level analysis, may not include all fluoroquinolone-resistance–conferring mutations. This list was generated by reviewing the current literature and available databases. The list of specific loci, and their association with fluoroquinolone resistance in *M. tuberculosis*, is constantly evolving. Third, there can be variability in MIC determination. However, each fluoroquinolone-resistant isolate was tested in triplicate by REMA, and we used the standard ofloxacin cutoff of 2 mg/L to determine fluoroquinolone resistance.

A strength of this study was that clinical *M. tuberculosis* isolates were studied, and thus of greater clinical relevance than laboratory strains. The study also evaluated the entire genome and utilized lineage-matched ofloxacin-susceptible control

Table 6. Sensitivity and Specificity of Different Thresholds of Whole-Genome Sequencing Compared to Phenotypic Drug Susceptibility

	Threshold		
	8%	2.5%	0.8%
Sensitivity	69%	86%	91%
Specificity	100%	100%	91%

Table 7. Sensitivity and Specificity of Whole-Genome Sequencing (WGS; Predominant Mutation Allele Frequency) Compared to Phenotypic Drug Susceptibility Testing (DST; Reference Standard)

		Comparison of Sensitivities of the 8% and 2.5% Thresholds			
WGS at 2.5% Threshold		WGS at 8% Threshold			
		Resistant	Not Resistant	Total	*P*[a]
	Resistant	29	7	36	.016
	Not Resistant	0	6	6	
	Total	29	13	42	
		Comparison of Sensitivities of the 8% and 0.8% Thresholds			
WGS at 0.8% Threshold		WGS at 8% Threshold			
		Resistant	Not Resistant	Total	*P*[a]
	Resistant	29	9	38	.004
	Not Resistant	0	4	4	
	Total	29	13	42	
		Comparison of Sensitivities of the 2.5% and 0.8% Thresholds			
WGS at 0.8% Threshold		WGS at 2.5% Threshold			
		Resistant	Not Resistant	Total	*P*[a]
	Resistant	36	2	38	.5
	Not Resistant	0	4	4	
	Total	36	6	42	

Abbreviations: DST, drug susceptibility testing; WGS, whole genome sequencing.

[a]*P* value derived from McNemar's test.

isolates to increase the likelihood of identifying SNPs associated with fluoroquinolone resistance.

In summary, current whole-genome sequencing analyses are insufficiently sensitive to detect fluoroquinolone resistance in *M. tuberculosis*, including in heteroresistant clinical isolates. Our study showed that use of the predominant mutation allele frequency in DNA gyrase, in conjunction with whole-genome sequencing, maintains high specificity and also improves the sensitivity to detect fluoroquinolone resistance in *M. tuberculosis*. Future studies to evaluate the 2.5% threshold as the optimal threshold to detect fluoroquinolone resistance in a more diverse sample are warranted.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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