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Genomic Analysis of the Evolution of Fluoroquinolone Resistance in *Mycobacterium tuberculosis* Prior to Tuberculosis Diagnosis

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Fluoroquinolones (FQs) are effective second-line drugs for treating antibiotic-resistant tuberculosis (TB) and are being considered for use as first-line agents. Because FQs are used to treat a range of infections, in a setting of undiagnosed TB, there is potential to select for drug-resistant *Mycobacterium tuberculosis* mutants during FQ-based treatment of other infections, including pneumonia. Here we present a detailed characterization of ofloxacin-resistant *M. tuberculosis* samples isolated directly from patients in Taiwan, which demonstrates that selection for FQ resistance can occur within patients who have not received FQs for the treatment of TB. Several of these samples showed no mutations in *gyrA* or *gyrB* based on PCR-based molecular assays, but genome-wide next-generation sequencing (NGS) revealed minority populations of *gyrA* and/or *gyrB* mutants. In other samples with PCR-detectable *gyrA* mutations, NGS revealed subpopulations containing alternative resistance-associated genotypes. Isolation of individual clones from these apparently heterogeneous samples confirmed the presence of the minority drug-resistant variants suggested by the NGS data. Further NGS of these purified clones established evolutionary links between FQ-sensitive and -resistant clones derived from the same patient, suggesting *de novo* emergence of FQ-resistant TB. Importantly, most of these samples were isolated from patients without a history of FQ treatment for TB. Thus, selective pressure applied by FQ monotherapy in the setting of undiagnosed TB infection appears to be able to drive the full or partial emergence of FQ-resistant *M. tuberculosis*, which has the potential to confound diagnostic tests for antibiotic susceptibility and limit the effectiveness of FQs in TB treatment.

The global emergence of drug-resistant tuberculosis (TB) has compelled the development of novel drugs and treatment regimens to combat this disease, which continues to kill an estimated 1.5 million people each year (1). The later-generation fluoroquinolones (FQs), particularly moxifloxacin, have shown a great deal of promise in animal models (2) and are currently recommended by the WHO as second-line drugs for the treatment of drug-resistant TB (3); however, they are not currently licensed for the treatment of drug-sensitive TB. Recently, three separate clinical trials evaluated a potential role for FQs in shortening the duration of therapy for drug-sensitive TB from the current 6 months to 4 months, an important goal aimed at simplifying TB therapy and reducing default rates, which contribute to the emergence of drug resistance (4–6). While the tested FQ-containing regimens showed superior rates of culture conversion, they were disappointingly not noninferior based on the rates of relapse and treatment failure (4–6). However, a phase 2b trial of a radically different regimen containing moxifloxacin, PA-824, and pyrazinamide recently reported bactericidal activity superior to that of standard therapy in drug-susceptible tuberculosis during the first 8 weeks of treatment (7). Thus, despite some setbacks, FQs continue to be explored as potential first-line drugs for the treatment of TB.

Because of their broad spectrum of activity, FQs are used in the treatment of a range of bacterial infections, including community-acquired pneumonia (8). FQs can also be highly effective in treating other conditions, such as urinary tract infections, skin infections, and sexually transmitted diseases, though their efficacy may be diminishing due to increasing resistance (9). Since FQ resistance can be fairly easily acquired through *de novo* mutation acquisition in its targets (10), FQ monotherapy can apply selective pressure that can lead to the emergence of resistant bacteria. Importantly, because of the broad spectrum of activity of FQs, selective pressure extends beyond the targeted etiological agent of disease to include commensals, transient colonizers, and possibly unrecognized coinfecting pathogens.

The broad-spectrum activity of fluoroquinolones is due to their ability to inhibit DNA replication in a broad range of species by targeting DNA gyrase and/or topoisomerase IV (10). In *Mycobacterium tuberculosis*, resistance to FQs is almost universally as-

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sociated with mutations in either of the two DNA gyrase subunits encoded by *gyrA* and *gyrB* (11, 12). Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* determine the vast majority (>80%) of clinically relevant FQ resistance (12, 13); *gyrB* mutations are often found in conjunction with *gyrA* mutations, playing an additive role. Single mutations corresponding to certain residues of GyrA, especially the aspartate at position 94, are sufficient to provide clinically relevant and diagnostically detectable levels of resistance. Other mutations within the QRDRs of *gyrA* and *gyrB* are associated with smaller increases in MIC that may not be detected using current culture-based assays (12).

Because of their role as second line-drugs, resistance to fluoroquinolones occurs most commonly in multidrug-resistant (MDR) TB (defined as resistant to the first-line drugs isoniazid [INH] and rifampin [RIF]). The WHO estimates that the proportion of MDR TB cases with resistance to an FQ was 21% in 2015 (1). FQ resistance in drug-sensitive TB is less common, being estimated at approximately 1% (14). Heteroresistance, the coexistence of sensitive and resistant organisms in the same patient, has recently been shown to be surprisingly common in FQ-resistant *M. tuberculosis* isolates (15); 38% of FQ-resistant isolates collected in Tennessee contained both sensitive and resistant bacteria (16). Also surprisingly, these heteroresistant samples were sensitive to all first-line TB drugs, suggesting that the emergence of FQ-resistant variants in these patients was not due to FQ use as second-line TB treatment.

We used next-generation sequencing (NGS) to sequence the genomes from a set of *M. tuberculosis* samples from Taiwan isolated directly from patients that were reported to be phenotypically resistant to ofloxacin (OFX), including isolates from patients who had not received an FQ for TB treatment. We found evidence of both heteroresistance to fluoroquinolones and the coexistence of subpopulations with different resistance mutations within the same isolate. By isolating and sequencing individual clones from these samples, we show a close evolutionary relationship between FQ-sensitive and -resistant clones derived from the same patient, suggesting the *de novo* emergence of FQ-resistant TB within these patients. This result suggests that selective pressure during FQ monotherapy in the setting of undiagnosed TB infection may be sufficient to drive the full or partial emergence of FQ-resistant *M. tuberculosis*. These findings have potential implications both for the use of FQs as first-line therapy for TB and for the ability of new molecular diagnostics to accurately predict FQ resistance.

MATERIALS AND METHODS

Strains and media. *M. tuberculosis* isolates were obtained from Lowenstein-Jensen slant cultures of sputum from HIV-negative patients with pulmonary TB in northern Taiwan from 2005 to 2011. Isolates were identified with standard biochemical tests. *M. tuberculosis* was cultured in Middlebrook 7H9 broth supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% oleic acid-albumin-dextrose-catalase (OADC) or on Middlebrook 7H10 agar supplemented with OADC and 0.5% glycerol. For NGS, archived isolates were minimally subcultured in the absence of antibiotic prior to being shipped to the Broad Institute as frozen glycerol suspensions. Ten-milliliter cultures were inoculated with 200 μl of this material and expanded to mid-log phase in antibiotic-free medium, which generally took 5 to 8 days, depending on the concentration of the inoculum and growth rate of the strain. All possible steps were taken to ensure that cultures from which DNA was extracted were grown for a minimal time required to obtain sufficient DNA for library construction. Cultures for DNA analysis of primary samples were always grown in the absence of OFX.

MIC determination. The susceptibilities of these isolates and clones derived from these isolates to isoniazid, rifampin, and ofloxacin were evaluated by the agar proportion method (APM) as described by the National Committee for Clinical Laboratory Standards (17). Critical concentrations were as follows: INH, 0.2 and 1 μg/ml; RIF, 1 μg/ml; OFX 2 μg/ml. MICs of clones of isolates to INH, RIF, and OFX were measured in quadruplicate in microtiter plates across a range of 2-fold dilutions of antibiotic in Middlebrook 7H9 medium supplemented as described above using a starting inoculum with an optical density at 600 nm (OD_{600}) of 0.02. The highest concentrations assayed were 3.2 μg/ml for INH, 0.32 μg/ml for RIF, and 32 μg/ml for OFX. After 10 days of unshaken growth at 37°C, wells were mixed using a multichannel pipettor and growth was measured by OD_{600}. The MIC was defined as the lowest concentration of antibiotic inhibiting growth relative to that in untreated control wells by >90%.

Whole-genome sequencing of isolates. Genomic DNA was prepared from heat-inactivated *M. tuberculosis* using a cetrimide-based protocol (18). For initial next-generation sequencing (NGS) analysis, fragment-based and/or jump-based paired-end genomic libraries were constructed and sequenced at the Broad Sequencing Platform using Illumina HiSeq, with a 101-bp read length. For individual OFX-sensitive and OFX-resistant clones, libraries were constructed using Nextera library construction kits (Illumina). Briefly, 50 ng of gDNA was input into Nextera library construction, and tagmented DNA was purified using DNA Clean & Concentrator-5 (Zymo Research). The purified tagmented DNA was amplified by index primers via a limited-cycle PCR and further purified with AMPure XP beads to remove very short library fragments. The quality of the library was checked by running 1 μl of 1:3-diluted library on an Agilent Technologies 2100 Bioanalyzer. DNA libraries were sequenced on MiSeq to produce 75-bp paired-end reads.

Reads were aligned to H37Rv (GenBank accession number NC_000962.3) using BWA version 0.5.9. Pilon (19) was then used to call variants with the –vcfqe option to produce quality-weighted read depths for each base at each position. To determine the fraction of reads corresponding to a specific base at a specific position, the quality-weighted read depth of a single base was divided by the total quality-weighted read depth at that position. Single-nucleotide polymorphism (SNP) calls derived from highly repetitive regions were excluded from downstream comparative analyses.

Sanger sequencing of *gyrA* and *gyrB* by PCR. Colonies picked from solid medium were cultured to OD_{600} of 0.5 to 0.8. A 50-μl aliquot was heat inactivated at 80°C for 2 h to be used as the template for PCR. PCR was performed using primers gyrBAseq 5′ (CGTAAGGCACGAGAGTTGGT) and gyrBAseq 3′ (GTTTTGTAGGCATCAGCGGT), which amplify a 1,539-bp region of the *gyrBA* locus containing the quinolone resistance-determining regions (QRDRs) of both *gyrA* and *gyrB*. The DNA sequences of *gyrA* and *gyrB* were aligned with the sequences of the QRDRs of *gyrA* and *gyrB* in *M. tuberculosis* strain H37Rv by using MacVector 5.0 software. The *gyrA* gene in all isolates varied from the H37Rv reference at 3 codons (E21N, S95T, and G668D); these are lineage-specific polymorphisms that are not associated with FQ resistance.

Accession number(s). Sequence information from this project is available via the NCBI website under BioProject number PRJNA191021.

RESULTS

Sample collection and sequence-based determination of ofloxacin resistance mechanism. We characterized five samples obtained from sputa from four patients seen at the National Taiwan University Hospital (Table 1) and diagnosed with culture-positive TB that was determined to be FQ (OFX) resistant based on the agar proportion testing. These were obtained from a collection which included samples from a 2007 study (20) and were selected based on initial PCR-based data suggesting that they did not carry

TABLE 1 Patient and sample information

Patient	Isolate	Days after initial visit	Resistance profile	OFX MIC for original isolate	Sanger sequencing result	NGS *gyrBA* genotype[a]	% colony formation on 2 μg/ml OFX
1	OFXR2	0	OFX monoresistant	2	WT	*gyrA* A90V (GCG→GTG: 1.9, 14/728)	0.8
2	OFXR11	0	OFX, low-level INH	4	WT	*gyrA* S91P (TCG→CCG: 2.8, 14/481), *gyrA* D94N (GAC→AAC: 0.8, 4/496), *gyrA* D94G (GAC→GGC: 1.2, 6/490)	1.4
3	OFXR14	0	OFX monoresistant	4	WT	*gyrA* A90V (GCG→GTG; 59, 304/208), *gyrB* S486F (TCC→TTC: 17, 71/359)	0.11 (9.8 on 1 μg/ml)
4	OFXR16	0	OFX monoresistant	8	D94G[b]	*gyrA* D94G (GAC→GGC: 68, 290/136), *gyrA* D89N (GAC→AAC: 3, 11/460), *gyrA* A90V (GCG→GTG: 1, 5/461), *gyrA* D94Y (GAC→TAC: 1, 4/429)	68
	OFXR15	76	OFX monoresistant	8	WT	*gyrA* D94G (GAC→GGC; 1.7, 10/592)	8

[a] The codon change, percentage of reads indicating the mutation, and number of mutant reads/number of WT reads are given in parentheses.
[b] There was evidence for the presence of the WT allele in the Sanger trace.

resistance-conferring mutations in the *gyrBA* locus and thus might possess a yet-uncharacterized FQ resistance mechanism. Three of these samples (OFXR2, OFXR11, and OFXR15) were phenotypically resistant to ofloxacin yet continued to possess wild-type (WT) *gyrA* and *gyrB* alleles (Table 1), while two others (OFXR14 and OFXR16) had ambiguous Sanger sequencing results (see Fig. S1 in the supplemental material), with traces suggesting the possible presence of both wild-type and resistant alleles of *gyrA*. Samples OFXR15 and OFXR16 were derived from the same patient, with sample OFXR15 collected 76 days after sample OFXR16. None of these 4 patients had received FQ antibiotics as a component of their TB treatment.

We sequenced the genomes of OFXR2, OFXR11, and OFXR15, along with the rest of the sample set for comparison, in an attempt to reveal the molecular basis of OFX resistance in OFXR2, OFXR11, and OFXR15. All samples were expanded in antibiotic-free medium prior to genomic DNA preparation and sequencing library construction. Illumina sequencing reads of 36 bp were aligned to a H37Rv reference genome, and all samples had coverage of >98% (Table 1).

Analysis of the Illumina sequence data using the Pilon SNP-calling algorithm failed to call any known FQ resistance-associated SNPs in OFXR2, OFXR11, or OFXR15. However, Pilon is a tool designed for the analysis of clonal samples, and mixed Sanger sequencing results from samples 14 and 16 suggested that heteroresistance might be confounding the analyses. We thus manually curated the Pilon output, examining the *gyrA* and *gyrB* region for evidence of reads suggesting heteroresistance. This review of the NGS data from OFXR2, OFXR11, and OFXR15 revealed that a small fraction of aligned Illumina reads (1.7 to 2.8%) from these samples appeared to contain known FQ resistance-associated SNPs (Table 1). Interestingly, further examination of the sequence data for FQ-resistant samples in which we had already identified a likely *gyrA* mutation, OXFR14 and OXFR16, also revealed the possible presence of *gyrA* alleles other than the dominant resistance-conferring allele that we had identified by a PCR-based Sanger analysis. The read depths at these positions ranged from 495 to 728, enabling us to detect potential minority populations as small as 1%; allele frequencies lower than 1% are challenging to identify due to the error rates intrinsic to Illumina sequencing (21).

As suggested by Sanger sequencing, an abundance of reads from OFXR14 and OFXR16 corresponded to resistance-conferring alleles of either *gyrA* or *gyrB*, but these could account for only 70 to 80% of the total reads, suggesting the possible continued presence of FQ-sensitive clones in these patients. These samples were each obtained from separate patients diagnosed with otherwise drug-sensitive TB and with no history of FQ use for the treatment of TB. In OFXR14, 59% of aligned NGS reads at nucleotide position 269 of *gyrA* corresponded to the *gyrA* A90V allele (GCG→GTG), while 16% of aligned NGS reads at position 1457 of *gyrB* indicated the presence of *gyrB* S486F (TCC→TTC). Due to the short nature of the Illumina sequencing reads (101 bp), we were unable to directly determine from the NGS data if these two mutation were present in the same genome or if they represented separate subpopulations, each carrying a different resistance-conferring mutation. In OFXR16, the majority of reads (68%) indicated the *gyrA* D94G allele (GAC→GGC), but small fractions of reads suggested the presence of other resistance-associated alleles, i.e., *gyrA* D89N (GAC→AAC, 3%), *gyrA* A90V (GCG→GTG, 1%), and *gyrA* D94Y (GAC→TAC, 1%). In this case, these different mutations were spaced closely enough to be potentially captured on the same Illumina read; however, visualization of reads across this region in Artemis showed that no read contained multiple SNPs, suggesting that multiple, independently arisen *gyrA* mutants might be present in the sample.

Purification and characterization of clones from heteroresistant samples confirm the presence of *gyrA* and *gyrB* alleles suggested by NGS. To confirm that the NGS reads reflected the genuine presence of minority populations of FQ-resistant and/or FQ-sensitive bacteria, we isolated individual clones from the original patient samples OFXR2, OFXR11, OFXR14, OFXR15, and OFXR16 on medium with or without OFX at 2 μg/ml, the critical concentration used in clinical diagnostics. The suspected heteroresistant samples yielded resistant colonies on OFX medium at rates generally reflecting the abundance of Illumina reads corresponding to resistant alleles (Table 1), while controls (H37Rv and an FQ-sensitive clinical isolate) yielded FQ-resistant colonies at frequencies of less than 1 in 10^7. Colonies were picked from both OFX-containing plates and drug-free plates to isolate individual clones, which were expanded in OFX-free medium for MIC determination and PCR sequencing of the QRDRs of *gyrA* and *gyrB*.

Indeed, the FQ-resistant clones derived from samples OFXR2,

OFXR11, and OFXR15, the samples which NGS data suggested contained only small subpopulations carrying a resistant allele, possessed the resistant genotypes predicted by the NGS data. Each of 10 resistant clones that we isolated from OFXR2 carried the identical C→T mutation leading to the common *gyrA* A90V allele, which confers moderate levels of resistance. Similarly, 5 of 5 FQ-resistant clones isolated from OFXR15 had the predicted A→G mutation leading to a *gyrA* D94G substitution, which is associated with high-level resistance.

The NGS data for OFXR11 suggested a more complicated situation that included the potential presence of 3 different resistance-conferring alleles, each in very low abundance (1 to 3%). The genuine presence of each of these alleles was confirmed in our PCR-based sequence analyses of isolated clones. The initial 11 clones isolated on 2 μg/ml OFX consisted of 7 D94N (GAC→AAC) mutants and 4 D94G (GAC→GGC) mutants. Surprisingly, despite the fact that reads corresponding to S91P (TCG→CCG) mutations were twice as abundant as the D94 mutations in the original Illumina sequencing data, no S91P alleles were identified in these 11 isolated clones. We suspected that this absence could have resulted from the isolation of clones on 2 μg/ml OFX, since the S91P mutation is associated with lower levels of resistance. We thus repeated the isolation at a lower OFX concentration (1 μg/ml). Under these conditions, S91P mutants indeed outnumbered those with mutations at D94, by a count of 81 to 7; the higher-than-anticipated ratio is likely due to an intentional bias toward smaller colonies during colony picking under the assumption that the less-resistant S91P mutants may be slightly less fit than D94 mutants, even at this lower OFX concentration. Of note, each of the individual clones purified from OFXR11 carried only a single, but different, nucleotide substitution in *gyrA*, suggesting that FQ resistance emerged independently at least 3 times within the same patient.

For samples OFXR14 and OFXR16, we were able to identify clones carrying the resistance-conferring SNP indicated in the NGS data as well as the persistence of an OFX-sensitive subpopulation. As with OFXR11, isolation of resistant clones from OFXR14 was complicated by the differing resistance levels associated with the 2 different SNPs suggested by the NGS data. Plating OFXR14 on 2 μg/ml OFX yielded resistant colonies at a frequency of only 0.11%, despite 59% of NGS reads corresponding to the *gyrA* A90V SNP. PCR-based sequencing of 4 clones isolated on 2 μg/ml OFX revealed that these all carried a *gyrA* D94G (GAC→GGC) mutation, which confers high-level resistance. Notably, the presence of this SNP was not apparent in the NGS data, as its abundance (0.11%) places it below the threshold for detection by sequencing. The 2 FQ resistance-associated SNPs that were present and dominated the NGS data, *gyrA* A90V (GCG→GTG) and *gyrB* S486F, are not associated with high-level resistance, explaining the inability to isolate them on 2 μg/ml. Isolation of clones on the lower concentration of 1 μg/ml OFX indeed yielded clones carrying the *gyrA* A90V (GCG→GTG) mutation (15 of 15); however, *gyrB* S486F mutants continued to elude isolation, even at this lower concentration used for selection. We were, however, able to isolate *gyrB* S486F mutants from nonselective medium. Microtiter-based MIC measurements of these clones showed that they have only a 2-fold increase in MIC relative to that of clones carrying wild-type *gyrA* and *gyrB* alleles, a shift that is insufficient to allow colony formation at 1 μg/ml OFX. Again, all isolated clones carried only a single, albeit different, FQ resistance-conferring mutation, suggesting multiple independent selections.

Analysis of the primary NGS data for isolate OFXR16 suggested that it is composed predominantly of clones carrying the high-level-resistance-conferring *gyrA* D94G (GAC→GGC) SNP, with 68% of reads corresponding to this allele. In good agreement with the NGS data, analysis of clones isolated in the absence of OFX revealed that 66% of 165 colonies analyzed were indeed D94G mutants, while 30% carried the wild-type *gyrA* allele and the remaining 5% contained one of three other minor genotypes that were also suggested in the NGS data: *gyrA* D89N (GAC→AAC), *gyrA* A90V (GCG→GTG), and *gyrA* D94Y (GAC→TAC). Clones carrying the *gyrA* D89N and *gyrA* A90V SNPs displayed lower levels of OFX resistance than those with the dominant *gyrA* D94G allele. Once again, all clones had a single *gyrA* mutation.

The MICs of the various clones obtained from samples 2, 11, 14, 15, and 16 (Table 2) are consistent with previous reports of the association of these specific polymorphisms with FQ resistance (22). The *gyrA* D94N and D94G mutations provide high level resistance (16-fold or higher increases in MIC relative to that of wild-type clones from the same sample), while *gyrA* A90V mutants show only a 4-fold increase in MIC. The *gyrA* D89N, *gyrA* S91P, and *gyrB* S486F mutants all display only 2- to 4-fold increases in OFX MIC.

We also measured the MICs of the clones isolated from OFXR2, OFXR11, OFXR14, OFXR15, and OFXR16 to INH and/or RIF. Since FQ resistance rates are much higher among samples with resistance to first-line antitubercular drugs (1, 20), evidence of a broader MDR or extensively drug-resistant (XDR) pattern in the purified resistant clones would suggest that the FQ-resistant clones originated via superinfection. However, none of the FQ-resistant clones examined was INH or RIF resistant, with the exception of the FQ-resistant clones derived from OFXR11, all of which displayed the identical low-level INH resistance (0.4 μg/ml) that was seen in the unpurified parental sample as well as the purified FQ-sensitive clone from OFXR11 (Table 2).

Genomic characterization of colony-purified clones. To determine if the multiple, allelically distinct clones derived from a given patient sample were clonal in origin, we performed NGS on 16 clones derived from samples OFXR2, OFXR11, OFXR14, OFXR15, and OFXR16, representing the FQ-resistant genotypes observed in the original NGS data. To evaluate the relatedness of the clones, we identified genome-wide polymorphisms in each strain relative to a reference genome (H37Rv). For our analysis, we focused only on single-nucleotide polymorphisms, including only sites for which there was coverage in all genomes and excluding genomic regions of low complexity. As shown in Fig. 1A, our initial NGS of the nonclonal samples shows that OFXR2, OFXR14, and OFXR11, are phylogenetically distinct from one another and from samples OFXR15 and OFXR16, which are sequential samples from the same patient (OFXR16 was isolated first, with OFXR15 obtained 76 days later). However, when we compare the genetic distance between the clones derived from within a sample (Fig. 1B) to the distance between the sensitive clones derived from different samples (Fig. 1C), the individual clones purified from a single sample appear to be indeed clonally derived. The FQ-resistant clone derived from OFXR2 differs from the sensitive clone by just 4 SNPs across the entire genome, excluding the SNP responsible for FQ resistance. Likewise, the 3 FQ-resistant clones derived from sample OFXR11 differ from the sensitive clone at only 1

TABLE 2 Properties of clones isolated from original samples

Sample	Clone	MIC (μg/ml)		Genotype	
		OFX[a]	INH	*gyrA*	*gyrB*
OFXR-2	S1	0.5	0.05	WT	WT
	R1	4	0.05	A90V GCG→GTG	WT
OFXR-11	S1	0.5	0.2	WT	WT
	R1	16	0.2	D94N GAC→AAC	WT
	R2	16	0.2	D94G GAC→GGC	WT
	R3	2	0.2	S91P TCG→CCG	WT
OFXR-14	S1	0.5	0.05	WT	WT
	R1	4	0.05	A90V GCG→GTG	WT
	R2	1	0.05	WT	S486F TCC→TTC
OFXR-15	S1	0.5	0.025	WT	WT
	R1	8	0.025	D94G GAC→GCC	WT
OFXR-16	S1	0.5	0.05	WT	WT
	R1	8	0.05	D94G GAC→GGC	WT
	R2	2	0.05	D89N GAC→AAC	WT
	R3	4	0.05	A90V GCG→GTG	WT

[a] An MIC of 0.5 μg/ml indicates full susceptibility.

nucleotide, excluding the *gyrA* SNP that confers FQ resistance, and differ from each other only at *gyrA*. The six clones purified from samples OFXR15 and OFXR16, which were obtained from the same patient 71 days apart, differed from each other by a maximum of 2 SNPs, excluding the FQ resistance-conferring SNPs; the sensitive clones from samples OFXR15 and OFXR16 differ by a single SNP. The greatest divergence was seen among the clones isolated from OFXR14. The 2 FQ-resistant clones derived from this sample diverge from each other and an isolated sensitive clone by 15 to 20 SNPs. However, the divergence between the OFXR14 clones and clones derived from other samples was enormous. The sensitive clone derived from OFXR14 differs from the sensitive clones derived from OFXR2, OFXR11, OFXR15, and OFXR16 by no less than 1,208 SNPs (Fig. 1C). The slightly larger divergence among the 3 clones derived from OFXR14 suggests that patient 11, from whom sample 14 was derived, was potentially infected for many years; the *in vivo* mutation rate of *M. tuberculosis* has been estimated at 5.5×10^{-10} mutations/base pair/generation for recently transmitted TB (23), which would yield approximately 1 mutation/year. A chronic infection would allow for the development of a genetically diverse population within the patient, and exposure of this population to FQs could select for clones carrying not only gyrase mutations but also hitchhiking SNPs; coselection of these hitchhiking SNPs could increase the apparent rate at which mutations accumulate (24). In all cases, the small number of polymorphisms between clones from the same patient contrasts with the large number of polymorphisms between samples collected from different patients. This underscores the close evolutionary relationship between clones within the same patient, which presumably are all descendants of a founder FQ-sensitive infecting strain.

DISCUSSION

Although FQs are highly effective second-line drugs for treating drug-resistant TB and are being evaluated as first-line agents, concerns linger about whether the emergence of resistance to these drugs will quickly compromise their utility. In this work, we microbiologically and genomically characterized a set of Taiwanese TB samples to reveal novel insights into how FQ resistance emerges in the context of TB infection. This characterization not only provides insight into the rapid acquisition of resistance within patients exposed to FQs outside a TB regimen but also highlights important implications for current and future TB diagnostics.

The 5 samples analyzed in our work were obtained from 4 patients who had not received FQs for the treatment of TB but were nonetheless infected by OFX-resistant *M. tuberculosis*. Each of these patients carried mixed populations of sensitive and resistant organisms. Deep sequencing by Illumina showed evidence of multiple *gyrA* alleles in 3 of these patients, which is consistent with the observations of Eilertson et al. (16). By isolating clones from these apparently heteroresistant samples, we were able to confirm the simultaneous presence of multiple *gyrA* and *gyrB* alleles within a single patient, each conferring different levels of OFX resistance. In all cases, the resistant clones derived from the same patient carried a single resistance-conferring SNP. Unlike previous studies (16), where lower-resolution hybridization-based assays (spoligotyping and mycobacterial interspersed repetitive unit [MIRU] typing [25]) were used to suggest that the simultaneous presence of sensitive and resistant organisms was not due to mixed infection, we have employed NGS to more precisely define the relatedness of sensitive and resistant clones derived from a single sample. An SNP-based comparative analysis shows that these clones differ by as little as a single polymorphism, while the genetic distances between clones derived from different patients were orders of magnitude larger, strongly supporting the notion that these FQ-resistant subpopulations can arise as the result of *in situ*, *de novo* generation of resistance within the patient. Additionally, in several instances, multiple mutants with highly similar genetic backgrounds were observed arising in parallel within the same patient.

The consecutive samples from patient 12 (samples OFXR16 and OFXR15) illustrate the complexities of the emergence of FQ

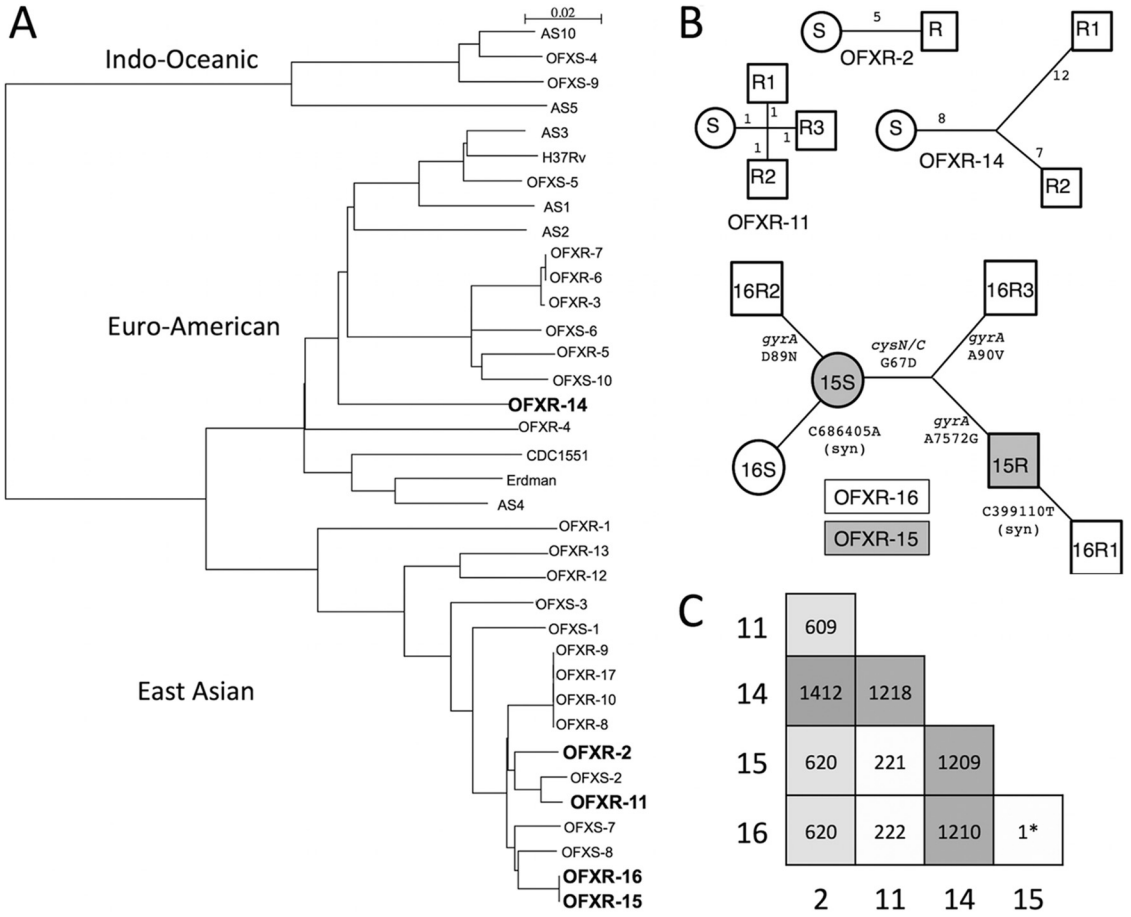

FIG 1 Relationships between clinical isolates and clones derived from those isolates. (A) SNP-based phylogeny of heteroresistant samples (bold) along with 22 regionally matched samples (OFXS-1 through OFXS-10 and OFXR-1 through OFX-17; 13 are OFX sensitive and 12 are OFX resistant), three laboratory strains (H37Rv, Erdman, and CDC1551), and six additional sensitive clinical isolates from the Broad Institute strain collection (AS1 to -5 and AS10). The tree was constructed using RAxML, the GTRCAT model, and 1,000 bootstrap replicates, using 8,625 sites with a passing SNP in at least one strain. (B) Graphical representation of the relatedness of individual colonies isolated from mixed samples. Circles indicate sensitive clones, and squares indicate resistant clones. The numbers along the branches indicate the number of high-confidence SNPs separating the samples or nodes from each other. Because they were obtained from the same patient, samples 15 and 16 are combined, and details of SNPs are shown, with numbering based on H37Rv genome coordinates. (C) Pairwise comparison of sensitive clones derived from the clinical samples OFXR2, OFXR11, OFXR14, OFXR15, and OFXR16, showing the number of positions that differ between the two strains, with darker shading indicating a greater number of differences. *, samples were derived from the same patient.

resistance within a patient, including the existence of multiple, unfixed *gyrA* mutations within a single patient sample. OFXR16 was the first sample obtained from patient 12 and contained several *gyrA* alleles, dominated by *gyrA* D94G, which was found in 66% of the clones characterized from this sample. Other alleles conferring lower-level resistance (*gyrA* D89N and *gyrA* A90V) were also found, albeit at just 1 to 2% of the total population; meanwhile, 30% of the clones remained FQ sensitive. As described above and shown in Fig. 1B, the various resistant and sensitive clones derived from OFXR16 were nearly identical, demonstrating that FQ resistance had arisen *in situ* in this patient. Intriguingly, 76 days later, a second sample, OFXR15, was obtained from this same patient (patient 12). In contrast to the case for the earlier sample, NGS data from this second sample suggested the presence of a much lower abundance of FQ-resistant bacteria. The *gyrA* D94G mutation, which had been present in 68% of the reads in the first sample, OFXR16 (66% of isolated clones), was now present in only 1.7% of the NGS reads from OFXR15; no evidence of the D89N or A90V allele was seen at all in the NGS data. Plating this isolate on 2 μg/ml OFX reduced the number of colonies by 99%, consistent with the much lower abundance of resistant genotypes found in the NGS data for this second isolate. The 5 colonies isolated on 2 μg/ml OFX all carried, as expected, the *gyrA* D94G allele.

During the 76-day interval between sample collections, this patient was not given an FQ for TB treatment, and thus there was no selective pressure promoting the new emergence of *gyrA* mutants or maintenance of the already present *gyrA* mutants. The large drop in the proportion of FQ-resistant bacteria present (from 68% to 1 to 2%) suggests that there may be an *in vivo* fitness cost associated with the *gyrA* mutations found in the primary sample. Alternatively, *in situ*-derived drug-resistant clones could remain relatively localized to particular lesions or regions of the lungs after they emerge, and the drop in the fraction of FQ-resistant bacteria seen in the second sample might reflect the differential progression or regression of lesions in spatially distinct regions of the lung, which would affect the source of the bacteria found in the sputum. Patient 12 was the only patient from whom we ob-

tained sequential samples, so a more complete assessment of whether *gyrA* mutants are generally less fit or whether other factors may have contributed to the lower proportion of FQ-resistant bacteria in the second isolate will require additional samples.

Because FQs were not used to treat these patients after the diagnosis of TB was made, the selection pressure that led to the emergence of these mutants within these patients was likely applied through the use of FQs prior to the diagnosis of TB. Although we cannot rule out the possibility that these patients acquired their infections from a contact who had previously been treated for TB with fluoroquinolone, this explanation seems unlikely. It is not standard practice in Taiwan to administer a fluoroquinolone as a first-line treatment for TB, and since the FQ-resistant clones derived from samples 2, 14, 15, and 16 are all INH and RIF sensitive, their source is unlikely to be a contact who had received or was receiving an FQ for TB.

FQs are used to treat a range of bacterial infections, including community-acquired pneumonia (26), a condition that can overlap in its clinical presentation with tuberculosis. A study conducted in Alberta, Canada, found that 17% of newly diagnosed TB patients had received one or more FQ prescriptions in the 6 months preceding their diagnoses (27). This likely led to periods of FQ monotherapy of their yet-undiagnosed TB, which can drive the emergence of FQ-resistant *M. tuberculosis* (28). Although previous reports, including this Canadian study, have shown that a single course of FQ is not associated with increased risk of FQ-resistant TB (26, 27), our genomic study suggests that this observation may be ignoring the existence of undetected low-frequency resistant subpopulations that could lead to FQ treatment failure. In fact, Long et al. observed that multiple FQ prescriptions (greater than 10 days of exposure) led to an 11-fold increase of FQ-resistant TB (27), supporting the notion that increased duration of FQ exposure promotes a more complete enrichment of FQ-resistant *M. tuberculosis*, enabling detection by current diagnostic methods.

The described heteroresistance also provides a challenge to existing and future diagnostics. The coexistence of wild-type and mutant alleles provides a mixed signal that has the potential to confound both culture-based and molecular diagnostic assays (15), depending on the assay's ability to detect small resistant subpopulations amid a large, sensitive majority population. The current, standard clinical laboratory method for identifying resistance to FQs is the agar proportion method (APM). Because this method defines susceptibility based on a ≥99% decrease in colony formation on 2 μg/ml ofloxacin, it will fail to identify a resistant subpopulation that is present at <1% (29). Alternative liquid culture-based assays such as MGIT 960 are also used in the clinic (30) but may miss very small resistant subpopulations due to the assay format, which uses a 1:100 dilution of the inoculum into drug-free medium as a reference against which growth in the presence of the drug is compared.

Recently, molecular diagnostics are being used with increasing frequency to determine resistance based on genotype (16). These molecular assays include PCR amplification of part or all of *gyrA* and *gyrB*, followed by Sanger sequencing, or the commercially licensed MTBDR*sl* line probe assay (Hain Lifescience). Additionally, Cepheid, whose current rapid PCR-based Xpert MTB/RIF assay (31) has been successfully implemented in clinical settings globally (32), is developing an expanded assay (Xtend XDR) to detect mutations conferring resistance to FQs, INH, amikacin, and kanamycin (33). The specificity of molecular diagnostic methods is high; the presence of any of a small number of mutations in the QRDR of *gyrA* (codons 94, 90, 91, 89, and 88) is strongly associated with FQ resistance. However, the sensitivity of these assays is approximately 80% (34), possibly due to their limited ability to detect resistant subpopulations. Even direct Sanger sequencing of PCR-amplified resistance loci requires minor alleles to be present at greater than 10 to 15% of the population (35). Specialized coamplification strategies such as coamplification at lower denaturation temperature-PCR (COLD-PCR) that enable detection of minor alleles at a frequency of 2.5% have been developed (36).

Whole-genome sequencing, in contrast, is at least equal to current culture-based methods and is superior to existing molecular diagnostics because it can detect minor alleles at frequencies at or below 1% (21). The intrinsic error rate of the sequencing technology, which is in the range of 0.3% for Illumina (21), sets the lower limit of sensitivity; improvement over time will be required to increase the sensitivity of resistance detection. NGS is an increasingly common tool in clinical microbiology (37), where it already plays a valuable role in disease surveillance and outbreak tracking (38). There is growing interest in incorporating NGS into routine diagnosis, particularly with *M. tuberculosis* (39, 40), where it has the potential to both identify infecting organisms and provide information on drug resistance (41). Important considerations in the application of NGS are the need for analytical methods that acknowledge the potential significance of low-frequency allelic variants as well as ensure that depth of coverage is sufficient to detect such minor alleles; in this work, coverage across the QRDRs of our original samples was typically in the range of 400× to 500×, enabling us to detect multiple reads corresponding to the resistant alleles later confirmed to be present. Like all existing molecular methodologies, NGS provides genotypic, not phenotypic, information and thus depends on a complete knowledge of resistance-conferring mutations and their effect on antibiotic susceptibility.

Numerous efforts to apply NGS to large collections of drug-resistant strains to catalog all possible genotypes that can confer drug resistance are under way, and these include work to discover and characterize novel genotypes that confer resistance to different TB drugs. This study highlights the importance of examining all raw sequencing data for minor alleles if sequencing from non-colony-purified samples, since we show that phenotypic resistance can result from minor populations of bacteria carrying known resistance mutations rather than from the majority population. Based on our observations, such isolates may initially appear to have a yet-uncharacterized resistance mechanism in the majority population when in fact that population is drug susceptible.

The complex mixtures of FQ-resistant and FQ-sensitive bacteria that we have described in this cohort of patients shows that further efforts are needed to understand the impact of this heterogeneity on diagnosis of resistance as well as the implications of both prescription-based and over-the-counter FQ use in promoting the emergence of FQ-resistant subpopulations in patients with undiagnosed TB. If FQs are to have any lasting value for treatment of TB, efforts must be made to prevent the inadvertent selection of FQ-resistant *M. tuberculosis* through unnecessary or unsupervised use of FQs in patients with undiagnosed TB.

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