

## Sequence Analyses of Just Four Genes To Detect Extensively Drug-Resistant *Mycobacterium tuberculosis* Strains in Multidrug-Resistant Tuberculosis Patients Undergoing Treatment<sup>∇†</sup>

Silke Feuerriegel,<sup>1\*</sup> Helen S. Cox,<sup>2</sup> Nana Zarkua,<sup>3</sup> Hamraev A. Karimovich,<sup>4</sup> Kai Braker,<sup>5</sup> Sabine Rüscher-Gerdes,<sup>1</sup> and Stefan Niemann<sup>1</sup>

Research Center Borstel, National Reference Center for Mycobacteria, Parkallee 18, 23845 Borstel, Germany<sup>1</sup>;  
Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia<sup>2</sup>;  
Médecins Sans Frontières, Tashkent, Uzbekistan<sup>3</sup>; Ministry of Health, Nukus, Karakalpakstan,  
Uzbekistan<sup>4</sup>; and Médecins Sans Frontières, Berlin, Germany<sup>5</sup>

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The rapid detection of *Mycobacterium tuberculosis* isolates resistant to second-line drugs is crucial for the institution of appropriate treatment regimens as early as possible. Although molecular methods have successfully been used for the rapid detection of resistance to first-line drugs, there are limited data on mutations that confer resistance to second-line drugs. To address this question, we analyzed *Mycobacterium tuberculosis* strains resistant to ofloxacin ( $n = 26$ ) and to capreomycin and/or amikacin ( $n = 48$ ) from Uzbekistan for variations in target genes (*gyrA*, *gyrB*, *rrs*, and *tlyA*). Strains susceptible to ofloxacin ( $n = 49$ ) and capreomycin and/or amikacin ( $n = 39$ ) were included as controls. Mutations in *gyrA* or *gyrB* were found in 96% (25/26 strains) of the ofloxacin-resistant strains, while none of the susceptible strains displayed mutations in those two genes. The most common mutation occurred in *gyrA* at codon 94 (17/26 strains [65.4%]), followed by mutations at codons 90 and 91. Two strains showed a mutation in *gyrB*, at codons 485 and 543, respectively; both mutations have not been reported previously. The most frequent mutation in strains resistant to both amikacin and capreomycin was A1401G in *rrs* (34/40 strains [85.0%]). Three strains had mutations in *tlyA*, of which two (at codons 18 and 118) were associated with resistance to capreomycin alone. Overall, none of the 10 resistant strains (5 amikacin-resistant and capreomycin-susceptible strains) and none of the 39 susceptible control strains had mutations in the genes investigated. Our results clearly demonstrate the potential of sequence analyses of short regions of relatively few target genes for the rapid detection of resistance to second-line drugs among strains isolated from patients undergoing treatment for multidrug-resistant tuberculosis. The mechanisms that confer amikacin resistance in this setting remain unclear.

With 9.2 million new cases and 1.7 million deaths in 2006, tuberculosis (TB) remains one of the most serious infectious diseases worldwide (28). In many settings, the emergence and transmission of drug-resistant *Mycobacterium tuberculosis* strains further threaten TB control efforts. TB fails to respond to treatment with the standard first-line drugs isoniazid (INH) and rifampin (RIF; rifampicin) in patients infected with so-called multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains. MDR-TB is particularly difficult to treat because the second-line drugs used are less potent and more expensive (12).

Disease caused by extensively drug-resistant (XDR) *M. tuberculosis* isolates, defined as MDR with resistance to any fluoroquinolone and at least one of three injectable second-line drugs (amikacin [AMK], capreomycin [CM], or kanamycin), is associated with very poor treatment outcomes (9).

The rapid detection of microbial resistance to second-line drugs prior to and during treatment is therefore of essential importance for the implementation of increased infection control measures that may well limit the spread of TB to other patients.

Compared to conventional liquid medium-based drug susceptibility testing, which still takes about 7 to 10 days, beginning from the time that a positive culture is obtained (18), the detection of genetic variants which mediate resistance to certain antimicrobial agents represents a more rapid alternative. Tests for the detection of MDR *M. tuberculosis* that detect mutations in the *rpoB* and *katG/inhA* regions, which confer resistance to RIF and INH, respectively, are now commercially available (3, 8, 13).

Resistance to fluoroquinolones, such as ofloxacin (OFX), commonly used to treat MDR-TB is thought to be mediated by mutations (single nucleotide polymorphisms [SNPs]) in the target genes *gyrA* and, less frequently, *gyrB*, which encode the respective subunits of the DNA topoisomerase gyrase (24). Most mutations conferring resistance to quinolones are known to accumulate in a short discrete region of the *gyrA* and *gyrB* genes termed the quinolone resistance-determining region (QRDR) (2).

Resistance to the aminoglycoside AMK and to CM are as-

\* Corresponding author. Mailing address. Research Center Borstel, National Reference Center for Mycobacteria, Parkallee 18, 23845 Borstel, Germany. Phone: 49-4537-188274. Fax: 49-4537-188311. E-mail: address: sfeuerriegel@fz-borstel.de.

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266 MDR-TB patients (study period: oct. 2003 - febr. 2006)

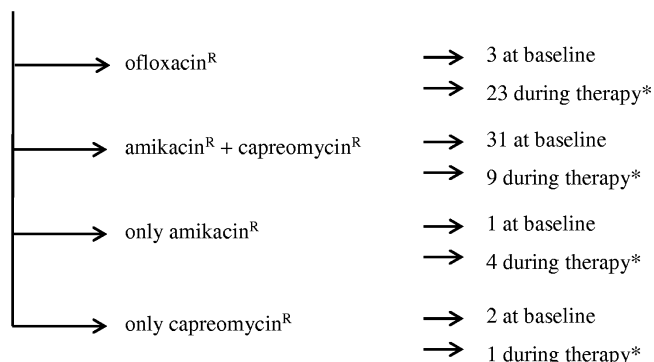


FIG. 1. Study design. Overall, 13 strains simultaneously showed resistance to OFX and injectable agents and were therefore XDR. Among the strains OFX resistant at the baseline, one also showed resistance to AMK and CM, whereas 12 strains which developed OFX resistance during therapy showed resistance to the injectable agents as well. Only one strain from the same patient was included and was either the baseline isolate or the first isolate found to be resistant at follow-up. R, resistance; \*, the isolates obtained during therapy were from both amplification (infection with the same strain) and reinfection cases.

sociated with SNPs in the 16S rRNA gene (*rrs*), especially in the region between nucleotides 1400 and 1500 (1, 23). Resistance to CM is thought to be additionally mediated by mutations located anywhere in the *thyA* gene, which encodes a 2'-O-methyltransferase (10, 15).

In the study described here, we aimed to determine if molecular analyses of target genes could be used for the rapid, specific, and sensitive detection of resistance to second-line drugs among new cases of TB and among previously treated patients in a high-incidence setting.

Sequence analyses of the genes associated with fluoroquinolone resistance (*gyrA* and *gyrB*) and aminoglycoside or CM resistance (*rrs* and *thyA*) were carried out with a panel of MDR- and XDR-TB strains arising from an MDR-TB treatment program in Karakalpakstan, a region in Uzbekistan with a high incidence of anti-TB drug resistance. Genetic data were then correlated with the results of phenotypic resistance testing.

#### MATERIALS AND METHODS

**Mycobacterial strains and growth conditions.** All mycobacterial strains used in this study were obtained from a program for the treatment of MDR-TB in Karakalpakstan, a region in the western part of Uzbekistan. The setting and treatment protocols employed have been described earlier (4). The region hosts a population of about 1.6 million people and is a high-incidence setting for TB (>120/100,000 population), with 13% of MDR-TB cases occurring among new cases of TB and 40% of MDR-TB cases occurring among previously treated patients (4).

Testing for susceptibility to first- and second-line drugs (INH, RIF, ethambutol, pyrazinamide, streptomycin, AMK, OFX, CM) was carried out as described earlier (7, 17, 21). For quality assurance, the laboratory participates in the external quality assessment program of the supranational laboratory network. Further details can be found in the supplemental material.

During the study period from October 2003 to February 2006, 476 strains from 266 MDR-TB patients were analyzed. Among these strains, 26 OFX-resistant isolates were identified; 3 were obtained from patients whose isolates had OFX resistance at the baseline (which means that the first isolate obtained already showed resistance) and 23 were obtained from patients whose isolates developed OFX resistance at some point during therapy. AMK and/or CM resistance was found in 48 strains; 34 of these strains were from patients whose isolates had

resistance at the baseline, and 14 were from patients whose isolates developed resistance during therapy (Fig. 1). Among these 48 strains, 5 were resistant to AMK but not to CM and 3 were resistant to CM but not to AMK.

Overall, 13 strains simultaneously showed resistance to OFX and injectable agents and were therefore XDR. Altogether, 149 strains (26 OFX-resistant strains and 48 injectable agent-resistant strains, of which 13 were XDR) were analyzed. Eighty-eight strains (either OFX or injectable agent susceptible) were randomly chosen as controls. Among this collection of strains, 49 strains were used as OFX-susceptible control strains and 39 were used as AMK- and CM-susceptible control strains.

**DNA isolation, PCR, and sequencing.** Genomic DNA was isolated as described previously (25). Sequencing was performed by a modified, nonradioactive mode of the dideoxynucleotide chain-terminating method of Sanger et al. (22) by using a commercial kit (Applied Biosystems, Darmstadt, Germany) and a 3130xl genetic analyzer. Details are described in the supplemental material.

#### RESULTS

Mutations were observed in the QRDRs of *gyrA* and *gyrB* in 25 of the 26 (96%) OFX-resistant strains, while none of the OFX-susceptible strains displayed mutations (Table 1).

The majority of OFX-resistant strains carried a mutation in a short hot-spot region of 15 bases in the *gyrA* gene ranging from codon 90 to codon 94 (23/26 strains [89%]), with mutations predominantly occurring at codon 94 (numbering of the codons is according to the TubercuList annotation). The most frequent single mutation detected was the substitution of adenine to guanine in *gyrA* at codon 94, which led to an amino acid change of aspartic acid to glycine (10/26 strains [38.5%]). Further mutations at codon 94 resulted in amino acid changes to alanine, asparagine, histidine, and tyrosine. All strains with mutations at codon 90 showed a change from cytosine to thymine, which led to an amino acid change of alanine to valine. At codon 91, a change of serine (TCG) for proline (CCG) was found in one strain. No double mutations were detected.

Only two OFX-resistant strains showed SNPs in *gyrB* exclusively; neither of these changes has been reported previously (Table 1). One of these strains had a change from cytosine to thymine, resulting in the replacement of Arg<sub>485</sub> with cysteine. The second strain carried a *gyrB* mutation at codon 543. This isolate showed both the wild-type sequence (Ala [GCG]) and the variant sequence (Thr [ACG]), suggesting that the speci-

TABLE 1. Mutations in the QRDRs of *gyrA* and *gyrB*<sup>a</sup>

Codon with <i>gyrA</i> or <i>gyrB</i> mutation, amino acid (nucleotide) change	No. (%) of strains:	
	Resistant to OFX	Susceptible to OFX
No mutation	1 (3.8)	49
<i>gyrA</i> 90, Ala (GCG) → Val (GTG)	5 (19.2)	
<i>gyrA</i> 91, Ser (TCG) → Pro (CCG)	1	
<i>gyrA</i> 94, Asp (GAC) → Gly (GGC)	10 (38.5)	
<i>gyrA</i> 94, Asp (GAC) → Asn (AAC)	1	
<i>gyrA</i> 94, Asp (GAC) → Ala (GCC)	4 (15.4)	
<i>gyrA</i> 94, Asp (GAC) → His (CAC)	1	
<i>gyrA</i> 94, Asp (GAC) → Tyr (TAC)	1	
<i>gyrB</i> 485, Arg (CGT) → Cys (TGT)	1	
<i>gyrB</i> 543, Ala (GCG) → Ala (GCG) + Thr (ACG)	1	
Total	26 <sup>b</sup>	49

<sup>a</sup> For the detection of OFX resistance, sequence analyses of *gyrA* and *gyrB* had a sensitivity and a specificity of 96.2 and 100%, respectively.

<sup>b</sup> Twenty-three strains were follow-up strains.

TABLE 2. Mutations in the resistance-determining region of *rrs* and in *thyA*<sup>a</sup>

<i>rrs</i> nucleotide change, and <i>thyA</i> codon, amino acid (nucleotide) change	No. (%) of strains:			
	Resistant to both AMK and CM	Resistant only to AMK	Resistant only to CM	Susceptible to AMK and CM
No mutation	5	5		39
<i>rrs</i> 1401, A → G	34 (70.8)			
<i>rrs</i> 1402, C → T			1	
<i>thyA</i> 18, Arg (CGA) → stop (TGA)			1	
<i>thyA</i> 118, Leu (CTG) → Arg (CGG)			1	
<i>thyA</i> 160, Leu (TTG) → Trp (TGG)	1			
Total	40 <sup>b</sup>	5 <sup>c</sup>	3 <sup>d</sup>	39

<sup>a</sup> Mixed infections were excluded from the analyses. The sensitivity and the specificity of the DNA sequencing of *rrs* and *thyA* for the detection of second-line injectable drug resistance were 79.2% and 100%, respectively.

<sup>b</sup> Thirty-one strains were resistant at the baseline.

<sup>c</sup> One strain was resistant at the baseline.

<sup>d</sup> Two strains were resistant at the baseline.

men may have been collected during the development of resistance during a period when the resistant variant has not outcompeted the susceptible bacilli, a phenomenon known as heteroresistance (20). Sequence analyses of *gyrA* and *gyrB* had a sensitivity and a specificity of 96.2 and 100%, respectively, for the detection of OFX resistance among the strains analyzed.

To determine the molecular basis of resistance to the second-line injectable agents AMK and CM, the *rrs* and *thyA* regions of 48 resistant strains and 39 susceptible strains were sequenced. Table 2 shows the mutations in the resistance-determining region of *rrs* and in *thyA* as well as the corresponding resistance phenotypes. The most common mutation detected was the change of adenine to guanine at position 1401 (34/48 strains [71.0%]) in *rrs* and was found only in strains resistant to both AMK and CM. One additional strain that was resistant only to CM displayed a C1402T mutation in *rrs*. Three resistant strains showed variations in *thyA*. The change of thymine to guanine at codon 160, which leads to an amino acid change of leucine to tryptophan, appears to confer resistance to both AMK and CM. Two SNPs, one at codon 18 (Arg [CGA] → stop [TGA]) and one at codon 118 (Leu [CTG] → Arg [CGG]), were found in strains resistant only to CM. The SNPs at codons 160 and 118 have not been described previously.

Among strains resistant to CM and/or AMK, 10 did not carry a mutation in *rrs* or *thyA*. Interestingly, all strains resistant to AMK alone ( $n = 5$ ) belonged to this group. Among the control group of 39 susceptible strains, none carried a mutation in either *rrs* or *thyA*. Thus, the sensitivity and the specificity of the DNA sequencing of *rrs* and *thyA* for the detection of second-line injectable drug resistance were 79.2% and 100%, respectively.

The practical adaptability of this method is demonstrated in an example in which the correlation between the mutations detected and phenotypic resistance for isolates taken from a single patient who developed XDR-TB during therapy is shown (see Table S2 in the supplemental material). Prior to treatment, the baseline strain was susceptible to OFX, CM,

and AMK but later developed resistance to all three drugs during therapy. No mutations were detected in the baseline isolate, while all three resistant isolates in follow-up samples carried the same mutation in *gyrA* at codon 94 and at position 1401 in *rrs*. The fact that all isolates had identical fingerprints (data not shown) demonstrates the development of resistance during treatment rather than reinfection in this case (5).

## DISCUSSION

Resistance to second-line drugs is a major factor mediating poor treatment outcomes in the treatment of drug-resistant TB. It is therefore essential to rapidly identify resistance to second-line drugs prior to and during treatment, in order to effectively guide treatment regimens. This study clearly demonstrates the potential of sequence analyses of short genomic regions to detect the major mutations mediating XDR-TB. In addition to known mutations, additional variations involved in second-line drug resistance in *M. tuberculosis* have been determined. Interestingly, the ability to confer resistance to second-line injectable agents appears to depend on the particular mutation, pointing to the possibility of resistance mutation-guided therapy.

Among the OFX-resistant strains investigated in the present study, the majority displayed SNPs in *gyrA* at codons 94 and 90. These mutations have previously been described as mediating resistance to quinolones (2). The different substitutions observed at position Asp94 in this study were also among those noted in fluoroquinolone-resistant strains of *M. tuberculosis* by Takiff et al. (24). In particular, the change to glycine at codon 94 occurs most frequently, probably because this mutation provides the greatest advantage for the cell concerning increased resistance and the least loss of fitness (24).

Mutations in *gyrB* occur less frequently than mutations in *gyrA*. However, two different mutations have been detected in this study, at codons 485 and 543, and neither mutation has been described previously. The strains with these two mutations exclusively carry the *gyrB* mutation and no additional mutation in *gyrA*. Previously, mutations in *gyrB* have been described to occur together with a *gyrA* mutation, leading to high-level resistance (11). However, single mutations in *gyrB* have recently been reported (19, 26, 27). While this study reaffirms the impact of mutations in *gyrA* on the development of fluoroquinolone resistance, the results suggest that mutations in *gyrB* should also be considered when one is screening for XDR strains.

Only one OFX-resistant strain analyzed had no mutation in the QRDR of either *gyrA* or *gyrB*. This strain possibly carries a mutation which is located outside the QRDRs, or the resistance may be caused by other mechanisms, such as enhanced drug efflux (16).

The most frequent mutation associated with resistance to both AMK and CM was found in *rrs* at nucleotide 1401. Cross-resistance to AMK, CM, and kanamycin has recently been described as being mediated by mutations in *rrs* (nucleotides 1401, 1402, and 1484) (14). The results of the analyses performed in this study support the existing data that the A1401G mutation in *rrs* actually mediates resistance to both AMK and CM.

However, our results clearly show that the resistance-con-



ferring potential of mutations in *tlyA* is dependent on the kind of mutation. As *tlyA* mutations have been detected in spontaneous CM-resistant *M. tuberculosis* mutants (15), the protein that is encoded has been proposed to be a drug target. In this study, one mutation at codon 160 was found in a strain resistant to AMK and CM, while mutations at codon 18 and at codon 118 were found exclusively in strains resistant to CM only. Thus, these mutations could be regarded as genetic markers for CM resistance, while it must be borne in mind that in the case of the strain resistant to both AMK and CM, a second mutation might confer resistance to AMK.

No mutation was detected in either *rrs* or *tlyA* in 10 strains, although these isolates were resistant to both AMK and CM or to AMK alone. Mutations outside the resistance-determining region are probably responsible for the resistance phenotype or, possibly, enhanced multidrug efflux (6). Interestingly, none of the strains which were resistant only to AMK carried a mutation in the regions of *rrs* and *tlyA* analyzed. These results point to so far undefined resistance mechanisms that mediate resistance to AMK alone and represent an interesting target for future studies.

In conclusion, sequence analyses of short regions of *gyrA*, *gyrB*, *rrs*, and *tlyA* showed a high sensitivity and a high specificity for the detection of resistance to second-line drugs among isolates from the study population. This allows the potential development of rapid PCR assays for the detection of second-line TB drug resistance among isolates from both new cases and previously treated patients.

The rapid detection of second-line TB drug resistance is of importance in guiding appropriate treatment regimens and in increasing infection control measures that may well limit the spread of TB.

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