

## Characterization of Tunisian *Mycobacterium tuberculosis* Rifampin-Resistant Clinical Isolates<sup>∇</sup>

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**Analysis of the gene encoding the  $\beta$ -subunit of *Mycobacterium tuberculosis* RNA polymerase (*rpoB*) has demonstrated a small region that harbors the mutations most frequently associated with rifampin resistance. In this study, we determined the occurrence of rifampin resistance in 544 Tunisian clinical *M. tuberculosis* strains isolated in a university hospital between 2004 and 2006 by using the standard-proportion agar method, the INNO-LiPA Rif.TB assay, and DNA sequencing.**

One of the most alarming trends concerning tuberculosis (TB) is the emergence of drug-resistant *Mycobacterium tuberculosis* strains, which has become a worldwide health care problem (15). The early detection of resistance to primary anti-TB agents is essential for the efficient treatment and control of multidrug-resistant (MDR) strains. Rifampin (RMP) is one of the most potent anti-TB drugs; therefore, resistance to RMP often results in high clinical relapse rates, particularly if RMP resistance is associated with resistance to other anti-TB drugs (8).

It has been established that RMP resistance in *M. tuberculosis* is mainly due to a group of mutations within a limited region of the *rpoB* gene that encodes the  $\beta$ -subunit of the RNA polymerase (28). These mutations can be characterized by PCR single-strand conformation polymorphism analysis (1, 23), heteroduplexing (21), dideoxy fingerprinting (6), the line probe assay (4, 7, 19), and automated DNA sequencing analysis (9, 24). However, few of these findings are associated with isolates from Tunisia, where the global incidence of TB infection was about 23.54 per 100,000 inhabitants (12). Therefore, the aim of our study was to determine the molecular basis of resistance in *M. tuberculosis* RMP-resistant strains by using the INNO-LiPA Rif.TB assay (Innogenetics, Ghent, Belgium) and DNA sequencing and to correlate these results with clinical and antibiotic sensitivity data.

A total of 544 clinical *M. tuberculosis* strains from 475 patients were isolated in a university hospital in an urban setting, the Rabta center, Tunis, Tunisia, during a 2-year period (2004 to 2006). The culturing of mycobacterial isolates was performed on solid Löwenstein-Jensen (LJ) medium. All *M. tuberculosis* cultures were biochemically characterized and confirmed by the AccuProbe method (Gen-Probe Inc., San Diego, CA).

Susceptibility testing for isoniazid (INH), RMP, ethambutol (EMB), streptomycin (SM), and ciprofloxacin (CIP) was carried out on LJ medium according to the standard procedure

(2, 3). The critical concentrations of RMP, INH, EMB, SM, and CIP were 40, 0.2, 1, 10, and 10  $\mu\text{g/ml}$ , respectively. Resistance to RMP was defined as  $\geq 1\%$  growth on RMP-containing medium compared to the rate of growth on control medium. On final analysis of the 544 isolates, 10 (1.83%) were RMP-resistant and 534 were characterized as fully susceptible to RMP. These 10 clinical isolates recovered from six different patients were classified as MDR strains since they were also resistant to INH. Complete medical records were available for all of the six patients, and these revealed negative human immunodeficiency virus status with previous anti-TB treatment histories, including RMP, INH, pyrazinamide, and SM during 3- to 6-month periods.

Susceptibility results for the other antituberculosis drugs tested showed 21 (3.8%) strains with a high level of resistance to INH, 99 (18.2%) resistant to EMB, 82 (15.07%) resistant to SM, and 5 (0.9%) resistant to CIP.

The template DNA for the molecular sequencing method was prepared by heat killing of mycobacteria at 95°C for 30 min, then sonication at room temperature for 25 min, and centrifugation at 14,000  $\times g$  for 5 min. Supernatant was kept at  $-20^\circ\text{C}$  until needed. The commercial PCR-based reverse-hybridization line probe assay (INNO-LiPA Rif.TB test; Innogenetics, Ghent, Belgium) using biotinylated primers (IP1 and IP2) was performed manually according to the manufacturer's instructions. The line probe assay consists of specific oligonucleotides immobilized at known locations on membrane strips and hybridized under strictly controlled conditions with the biotin-labeled PCR product. The LiPA results were evaluated as described elsewhere (5).

DNA sequencing of both strands was performed with an ABI PRISM Dye terminator cycle sequencing ready reaction kit (Applied Biosystems) and the reactions were analyzed on an ABI PRISM 310. The BLAST 2 SEQUENCES computer program was used for DNA sequence comparisons (<http://www.ncbi.nlm.nih.gov/BLAST>).

The presence of the *M. tuberculosis* complex was confirmed by a positive reaction with an oligonucleotide-specific probe for this complex. The results of the INNO-LiPA assay showed three different patterns with mutations in codon 526 or 531

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TABLE 1. Resistance patterns of RMP-resistant *M. tuberculosis* isolates by proportion method, LiPA, and DNA sequencing

Patient	Strain	Isolation date (day/mo/yr)	Drug resistance shown by proportion method	Mutation shown by:	
				LiPA	Sequencing <sup>a</sup>
1	10603	21/12/2004	INH, RMP, SM	ΔS5	S531W
	5585	12/05/2005	INH, RMP, SM, EMB	ΔS5	S531W
	4522	12/07/2006	INH, RMP, SM, EMB	ΔS5	S531W
2	15	03/01/2006	INH, RMP	R5 <sup>b</sup>	S531L
	3267	28/04/2006	INH, RMP	R5	S531L
3	9796	25/11/2005	INH, RMP, EMB, SM	ΔS4/ΔS5	H526C/S531A
	1409	28/02/2006	INH, RMP, EMB, SM	ΔS4/ΔS5	H526C/S531A
4	2603	03/03/2006	INH, RMP	R5	S531L
5	9742	23/11/2005	INH, RMP, SM, CIP	ΔS4/ΔS5	H526C/S531A
6	4164	29/05/2006	INH, RMP, EMB	ΔS5	S531W

<sup>a</sup> Sequencing mutation descriptions are as follows: first, the amino acid residue in the wild-type sequence, then the codon position, and finally the amino acid residue in the mutated sequence. The numbering system is based on the *Escherichia coli* β-subunit of RNA polymerase (25).

<sup>b</sup> R5, S531L.

leading to an amino acid change (Table 1). In all cases, we observed a single nucleotide mutation in codon 531, with substitutions of serine to tryptophan (TCG to TGG), serine to leucine (TCG to TTG) and serine to alanine (TCG to GCG). Three *M. tuberculosis* strains showed triple point mutations in two different codons (codon 526, His CAC to Cys TGC, and codon 531, Ser TCG to Ala GCG), which has not been previously reported. The compilation of data available from many studies indicated that RMP resistance in *M. tuberculosis* is mainly due to distinct mutations located within an 81-bp RMP resistance-determining region of the *rpoB* gene (8, 10, 11, 14, 16, 20, 26, 29). However, it is speculated that additional mechanisms, including a permeability barrier or membrane proteins acting as drug efflux pumps, may also be involved in conferring the resistance phenotype (7, 14, 17).

Worldwide, the most frequent mutations are S531L, H526Y, and H526D, and all three can be reliably detected and differentiated by the INNO-LiPA Rif.TB test (19). Significantly, the frequency of mutations was higher at codon 531 and lower at codon 526 in the Tunisian isolates in our study than in those described previously. Moreover, the *M. tuberculosis* RMP-resistant strains that we investigated showed no mutations in codon 516. Also, a lot of novel mutations involving changes in one or two codons were identified in different geographic areas (22, 27), and it remains to be seen if the new mutation identified in our study can also be found in other parts of Tunisia, based on an epidemiologically independent group of isolates.

In Tunisia, as is the case worldwide, the vast majority of *M. tuberculosis* RMP-resistant strains are also INH-resistant, and although monoresistance to INH is common (18), monoresistance to RMP is rare (13). Thus, RMP resistance can be used for the identification of MDR tuberculosis infections. Spontaneous mutations that lead to drug resistance occur rarely in *M. tuberculosis*, and multidrug regimens can prevent the emergence of clinical drug resistance (25). The ability to rapidly

identify an MDR-TB infection affords an effective management tool by reducing the frequency of noncompliance, since patients that do not have MDR-TB can be treated less aggressively, and this ability therefore contributes to the control of TB.

Despite the small number of strains investigated, the concordance between phenotypic RMP resistance susceptibility testing results and molecular sequencing results was 100%, indicating that the INNO-LiPA Rif.TB PCR-based hybridization assay is highly reliable when run in parallel with conventional laboratory TB diagnostics. The LiPA assay, though relatively expensive in low-resource countries, is simple and convenient. However, DNA sequencing is the "gold standard" for mutation detection, because it provides a definitive identification of any mutation present.

**Nucleotide sequence accession number.** The sequence with novel mutations found in this study has been deposited in GenBank under accession number EF064790.

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