Mutations in the *rpoB* Gene of Rifampicin-resistant *Mycobacterium tuberculosis* Strains Isolated in Brazil and France

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We evaluated the mutations in a 193bp of the rpoB *gene by automated sequencing of rifampicin (RMP)-resistant and susceptible* Mycobacterium tuberculosis *strains isolated from Brazil (25 strains) and France (37 strains). In RMP-resistant strains, mutations were identified in 100% (16/16) from France and 89% (16/18) from Brazil. No mutation was detected in the 28 RMP-susceptible strains. Among RMP-resistant or RMP-susceptible strains deletion was observed. A double point mutation which had not been reported before was detected in one strain from France. Among French resistant strains mutations were found in codons 531 (31.2%), 526, 513 and 533 (18.7% each). In Brazilian strains the most common mutations were in codons 531 (72.2%), 526 (11.1%) and 513 (5.5%). The heterogeneity found in French strains may be related to the fact that most of those strains were from African or Asian patients.*

Key words: *Mycobacterium tuberculosis* - *rpoB* gene - rifampicin-resistant - Brazil - France

Resistance of *Mycobacterium tuberculosis* to antituberculous drugs has emerged as a major public health threat (Pablo-Mendes et al. 1998). The short course therapy with rifampicin (RMP), isoniazid (INH) and pyrazinamid (PZA) is the most effective regimen against tuberculosis. The efficacy of this regimen is hampered in regions where there is a high initial resistance to RMP. In those regions, the rapid detection of RMP-resistance is urgently needed. New methodologies have been proposed to cover this situation.

Development of resistance to RMP in *M. tuberculosis* follows a "single-step" high-level resistance pattern (Morris et al. 1995, Musser 1995). Mutants arise spontaneously in strains not exposed previously to the antibiotic at a rate of one mutation per 10^{-7} to 10^{-8} organisms (Cole 1994, Williams et al. 1994). Resistance has been attributed to changes in structural RNA polymerase (Watterson et al. 1998, Yuen et al. 1999). The structural and functional organization of RNA polymerase is conserved among bacteria (Hunt et al. 1994). *Escherichia coli* resistance to RMP is associated with specific nucleic acid substitutions in the gene encoding for RNA polymerase subunit-β (*rpoB*) (Miller et al. 1994, Kapur et al. 1995). Mutations associated with the RMP-resistant phenotype are generally localized in a 69bp "hot spot" region with mutations frequently in the codons: Ser-531, His-526 and Asp-516 (Telenti et al. 1993a, Williams et al. 1994, Hunt et al. 1994, Kapur et al. 1995, Musser 1995). Variations of mutations in *rpoB* gene have been described in *M. tuberculosis* strains isolated from different regions of the world *(*Kapur et al. 1995, Morris et al. 1995, Whelen et al. 1995). In only 5% of RMP-resistant *M. tuberculosis* strains, mutations are not identified in a "hot spot" fragment; in these cases mutations at codons 381, 481, 505, 508, and 509 (Watterson et al. 1998) are sometimes reported. Commercial kits which cover the four most frequently observed mutations (D516V, H526Y, H528D and S531L) of the "hot spot" *rpoB* region have been proposed for clinical use (Inno-Lipa RifTB; Innogenetics, Belgium) (Goyal et al. 1997, Matsiota-Bernard et al. 1998, Hirano et al. 1999).

In France, the tuberculosis incidence is 17/100,000 inhabitants and resistance to anti-tuberculosis drugs has recently increased (Liard et al.

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1994, Salomon et al. 1994). In Brazil, tuberculosis incidence is higher in urban areas such as the States of Rio de Janeiro (120/100.000) and São Paulo (80/100.00). Since 1981, the Brazilian National Tuberculosis Control Program adopted the daily self-administered short-course treatment regimen (2 RMP + INH + PZA/4 RMP + INH). The urban areas have reached 28% leading to an increase of tuberculosis abandon (Kritski et al. 1996, 1998). In Rio de Janeiro, drug-resistance surveillance performed in outpatient clinic showed a level of 1% of RMP primary resistance, but in patients attended in hospital, it increases to 7% (Pablo-Mendez et al. 1998).

In this study, we evaluate mutations in the *rpoB* gene of RMP-resistant and susceptible *M. tuberculosis* strains isolated from Brazil (25 strains) and France (37 strains).

MATERIALS AND METHODS

Characterization of mycobacterial clinical isolates - Sixty two strains (34 RMP-resistant and 28 RMP-susceptible *M. tuberculosis* isolates) were recovered from Brazilian and French patients: Rio de Janeiro University, Brazil (14 strains), São Paulo University, Brazil (11 strains), France (13 strains), Africa (20 strains) and Asia (4 strains). One reference strain H37Ra was included.

Detection of rifampicin resistance - Strains were identified by standard biochemical methods in the Institut Pasteur of Lyon. RMP resistance was determined three times for each strain by the disk elution method using a 25 µg disk corresponding to 5 µg/ml final concentration (Sensi-Disk; BBL, Becton Dickinson Microbiology System, Cockeysville, MD).

Preparation of genomic mycobacterial DNA - Mycobacterial strains were cultured for three or four weeks at 37°C in 40 ml of Middlebrook 7H9 liquid medium with Dubos oleic albumen complex enrichment (Difco Laboratories, Detroit, MI) and 0.05% Tween 80. Cells were heat-killed at 80°C for 20 min, then harvested by centrifugation at 7,000 g for 30 min. Bacterial cells were resuspended in 10 mg/ml lysosyme in 10 mM Tris, 1 mM EDTA (pH 8.0) for 2 h at 37°C, then lysed by sonication (Ultrasonic, BioBlock, Scientific®-88169), 12 min at 40°C. Finally, lysate was incubated for 1 h at 60°C with 0.5 mg/ml proteinase K, 1% sodium dodecyl sulphate in 50 mM Tris, 100 mM EDTA (pH 7.5). The DNA was purified by phenol-chloroform-isoamylalcohol extraction and isopropanol precipitation. The pellet was washed twice in 70% ethanol, then resuspended in 50 mM Tris, 100 mM EDTA (pH 8.0), and 5 µl of 1/500 dilution of purified DNA in water was used as a target in all polymerase chain reaction (PCR).

Sequencing analysis - Primers for PCR were synthesized with an Expedite Nucleic Acid Synthesis System (Perspective Biosystem). PCR reactions (50 µl) contained target DNA (10 µg), 15 pmol primers, 20 mM dNTP (Pharmacia Biotech), 2.5U AmpliTaq Gold polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), and KCl 500 mM, Tris HCl 100 mM, $MgCl_2$ 15 mM, gelatin 0.1%, pH 8.3. The reaction was performed in a Cyclogene Tech. thermal cycler (USA).

Primers and amplification conditions for the tuberculosis *rpoB* locus were as follows: upstream primer (rpo 105) 5'-CGTGGAGGCGATCACACC GCAGACGT-3'; downstream primer (rpo 273) 5'-GACCTCCAGCCCGGCACGCTCACG-3', which produced a 193-bp amplicon (Kapur et al. 1994). Amplification was performed during 40 cycles of 96°C (1 min), 55° (1 min) and 72°C (30 sec), after an initial 5 min at 96°C to denature input DNA, with a final 10 min at 72°C for strand extension.

Amplification products were detected by agarose gel electrophoresis in 2% agarose (Seakem GTG; FMC, Rockland, ME, USA) made up in 0.5x Tris-Borate-EDTA (TBE), with ethidium bromide. The products were not purified, and were sequenced by using primer rpo105 and rpo273 with Applied Biosystems 373A or 377 automated sequence protocol (ABI PRISM^{TM} Dye Terminator).

Sequence alignment was done by using dedicated software (Villefranche Sur Mer, CNRS, Richard Christen).

Determination of minimal inhibitory concentration (MIC) - For one strain the MIC was determined by proportion agar dilution method. Middlebrook 7H10 agar, supplemented with Middlebrook OADC enrichement (DIFCO Laboratories, Detroit, MI) was used to prepare quadrant plates with different concentrations of drugs (Kent & Kubica 1985): RMP (0.004-40 µg/ml).

RFLP (restriction fragments length polymorphism) analysis - All the strains were analyzed in accordance with the protocol of Van Soolingen et al. (1992) for RFLP-IS6110.

RESULTS AND DISCUSSION

Among the Brazilian strains from Rio de Janeiro, 12 (85.7%) were RMP resistant and 2 (14.3%) RMP-susceptible. Among the strains from São Paulo, 6 (54.5%) were RMP-resistant and 5 (45.5%) RMP-susceptible. Among the strains from Institut Pasteur of Lyon, France, 16 (43.2%) were RMP-resistant and 21 (56.7%) RMP-susceptible.

The presence of point mutations in the *M. tuberculosis* RMP-resistant strains was confirmed in 32/34 (94.1%) strains, within an amplicon representing a 193-bp region of the *rpoB* gene (Table

I). The mutations in the RMP-resistant strains are demonstrated in the Table II; only two strains from Rio de Janeiro had no mutation. A double point mutation was detected in one strain from France, in two noncontiguous codons, but no insertion or deletion was observed (Table II).

In this study, the mutations within the *rpoB* gene of *M. tuberculosis*, which appear to be associated with the RMP-resistant phenotype identified, are consistent with other publications (Hunt et. al. 1994, Kapur et al. 1995, Kreiswirth & Musser 1995, Musser 1995). Point mutations within the Ser-531, His-526, Gly-513 or Asp-516 codons has been shown to lead to high-level resistance in *E. coli* (20 µg/ml) and *M. tuberculosis* (Jin & Gross 1988, Telenti et al. 1993a,b, Williams et al. 1994, Whelen et al. 1995). In our study, we found a change in codon 533 in four strains. This mutation have been reported for RMP-resistant and RMP-susceptible strains for Taniguchi et al. (1996). A double mutation in codons 516 (Asp) and 533 (Leu) was observed in our study (Gen Bank access number AF 292115), and presented a high level of resistance (MIC > 40 µg/ml). These data have not been reported before.

In Brazilian isolates, a predominance of point mutation was observed in codon 531. In French *M. tuberculosis* strains this figure did not occur. The variation observed in the French isolates may be due to the heterogeneity of the native countries of origin of the patients, as demonstrated in Table II. When we compared the RFLP profile with points mutation, no RFLP clustering was associated with mutations frequency described in other studies (Telenti et al. 1993b, Spindola de Miranda et al. 1996).

No point mutations were found in the RMP-susceptible strains in the sequenced region studied as previously described, but in Brazilian isolates, the absence of mutations in two RMP-resistant strains may be due to mutations outside the *rpoB* region studied or to another resistance mechanism. These results suggest the usefulness of the rapid determination of the drug-susceptible characterization of *M. tuberculosis* using automated DNA sequencing methods.

The results obtained in our study must be validated with a great number of RMP-resistant strains for different region in Brazil. The MICs analysis of *M. tuberculosis* would be useful to evaluate the relationship between resistant phenotype and points mutation (Ohno et al. 1996).

TABLE I

Different mutations observed in the region of 78pb for the gene *rpoB*

Codon	Frequency	Percentage
531 Ser TCG → Leu TTG	18	56.2
526 His CAC → Pro CCC	2	6.2
His CAC → Leu CTC	1	3.1
His CAC → Asn AAC	1	3.1
His CAC → Arg CGC	1	3.1
513 Gly CAA → Lys AAA	3	9.4
Gly CAA → Pro CCA	1	3.1
516 Asp GAC → Val GTC	1	3.1
533 Leu CTG → Pro CCG	3	9.4
516 Asp GAC → Gly GGC + 533 Leu CTG → Pro CCG	1	3.1
Total	32	100

TABLE II

Different mutations of rifampicin-resistant strains (gene *rpoB*) in accord with origin

Codon	Origin of strains (France)		Origin of strains (Brazil)		Total
531	France	2 (12.5%)	Rio de Janeiro	7 (38.9%)	
	Africa	2 (12.5%)	São Paulo	6 (33.3%)	18 (52.9%)
	Asia	1 (6.2%)			
526	France	1 (6.2%)	Rio de Janeiro	2 (11.1%)	5 (14.7%)
	Africa	2 (12.5%)			
516	Asia	1 (6.2%)		0 (-)	1 (2.9%)
513	France	1 (6.2%)	Rio de Janeiro	1 (5.5%)	4 (11.8%)
	Africa	2 (12.5%)			
533	Africa	1 (6.2%)		0 (-)	3 (8.8%)
	Asia	2 (12.5%)			
516+533	France	1 (6.2%)		0 (-)	1 (2.9%)
No mutations		0 (-)	Rio de Janeiro	2 (11.1%)	2 (5.8%)
Total		16 (100%)		18 (100%)	34 (100%)

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