A Simple and Rapid Technique for the Detection of Rifampin Resistance in *Mycobacterium leprae*[1]

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Rifampin is the key component of the standard multidrug regimen used for the treatment of leprosy ([5, 13]). It would be highly desirable to have at our disposal a rapid, yet simple, technique for monitoring suspected cases of rifampin resistance which could be applied directly to biopsy material. This could replace the extremely long rifampin-susceptibility test, currently performed after inoculation of mice ([11]), or the radiorespirometric method which requires relatively large numbers of metabolically active *Mycobacterium leprae* ([2, 3]). In a recent study, the molecular basis of rifampin resistance in *M. leprae* was established ([7]) by direct DNA sequence analysis of the *rpoB* genes, amplified by the polymerase chain reaction (PCR) ([14]) from drug-resistant isolates. Resistance was shown to result from a limited number of missense mutations located within a short stretch of the gene. Since direct sequencing of PCR products is a relatively laborious approach, the single-strand conformational polymorphism (SSCP) technique developed by Orita ([10]) for detecting point mutations and other polymorphisms has been adopted. When applied to the mutants characterized previously ([4, 7]), the *M. leprae* isolates could be classified as rifampin-resistant or -sensitive in less than 2 days. The method has been appraised by analyzing a new, putatively rifampin-resistant mutant of *M. leprae* isolated from a treated lepromatous leprosy patient who had relapsed.

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MATERIALS AND METHODS

***M. leprae* strains.** The nine rifampin-resistant mutants characterized in detail by Grosset, *et al.* ([4]) and analyzed recently by DNA sequencing ([7]) were again employed together with the five rifampin-susceptible isolates used previously as controls (The Table). A new strain, 92041 (The Table), was detected in biopsy material obtained from a lepromatous leprosy patient originally from Martinique but now resident in France. The patient had been treated with dapsone monotherapy from 1958–1977, and then with rifampin alone for 2 years before starting a course of rifampin plus prothionamide. The patient, who appears to have taken his medication irregularly, relapsed after some years and was suspected of harboring rifampin-resistant bacilli.

PCR procedures. The region of *rpoB* known to harbor mutations conferring rifampin resistance was amplified directly from "freeze-boiled" biopsies ([14]) by the polymerase chain reaction (PCR) using primers Brpo22 (CAGGACGTCGAGGCGATCAC) and rpo32 (TCCTCGTCAGCGGTCAAGTA), as described previously ([7]). This gave rise to a fragment of 390 bp which was uniformly labeled with ^{32}P by including 5 μCi of ($\alpha^{32}P$) dCTP (3000 Ci/mmole; Amersham International, Amersham, U.K.) in the PCR reaction which was performed using 35 cycles (1 min at 92°C, 2 min at 61°C and 2 min at 72°C) with a final elongation time of 10 min at 72°C. DNA sequences were obtained directly from unlabeled PCR products as described ([7]).

SSCP analysis. To detect mutations in the *rpoB* fragment, single-strand conformation polymorphism (SSCP) analysis was employed using the conditions described recently ([8, 12]). Briefly, 10 μl of the ^{32}P-labeled PCR fragment was mixed with 15 μl of H_2O and 25 μl of a solution containing 0.1% sodium dodecyl-sulfate (SDS) and 2 mM eth-

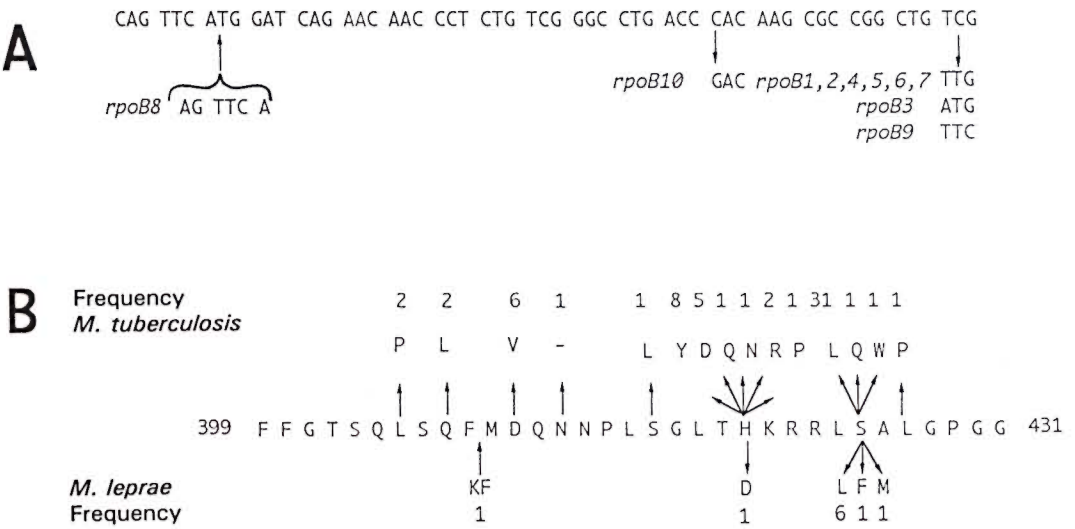

FIG. 1. **A.** Nucleotide sequence of a short region of *rpoB* from *M. leprae* harboring mutations conferring rifampin resistance. Base changes and corresponding alleles are indicated. **B.** Amino-acid sequence comparison of part of region II, of the β subunit of RNA polymerase from *M. tuberculosis* and *M. leprae*. Residue numbers are indicated together with the amino acids in the one letter code. Mutated amino-acid residues associated with rifampin resistance are shown along with the frequency with which a given mutation has been isolated. Data for *M. tuberculosis* were taken from ([8, 12]). One letter codes for common amino acids are as follows: A = alanine; C = cysteine; D = aspartic acid; F = phenylalanine; G = glycine; H = histidine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.

ylene-diamine tetra-acetic acid (EDTA). A 5 μl aliquot was mixed with sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 95°C for 10 min to denature the PCR fragment. Samples were quick-chilled on ice to prevent renaturation and immediately loaded onto a 5% polyacrylamide gel (30% acrylamide/0.5% bis-acrylamide; 20 × 50 cm) containing TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Electrophoresis was conducted for 2.5 hr at constant power (65W) using the TBE buffer. The gel was dried and subjected to autoradiography.

In some experiments, the size of the PCR fragment was reduced by digestion with restriction endonuclease *Pvu*I (10 μl PCR reaction, *Pvu*I buffer, 4 units of enzyme in a final volume of 25 μl) for 1 hr, thus yielding two fragments of 239 bp and 149 bp (the larger of which carries the mutations). This procedure enhanced the resolution of the strands on the gel and accentuated the differences due to the mutations. When the digestion was performed, samples were mixed directly with an equal volume of a solution containing SDS and EDTA and then processed as described.

RESULTS

It has been shown recently in nine independent isolates of *M. leprae*, that rifampin resistance is due to tightly clustered mutations in a short region of the *rpoB* gene ([7]). In seven cases, a single base change was found by DNA sequencing; in the eighth mutant two consecutive nucleotides deviated from the wild type *rpoB* sequence ([6, 7]). A 6 bp insertion was detected in the remaining mutant. These findings are summarized in Figure 1.

Since DNA sequencing is relatively time-consuming and labor-intensive, we decided to adapt the SSCP technique ([10]) in order to develop a simpler means of detecting mutations. Consequently, PCR was employed to amplify the region of *rpoB*, which has been found to be prone to mutation, from the nine resistant mutants studied previously and five susceptible strains. In addition, to test the power of the method, acid-fast bacilli (AFB) harvested from the skin

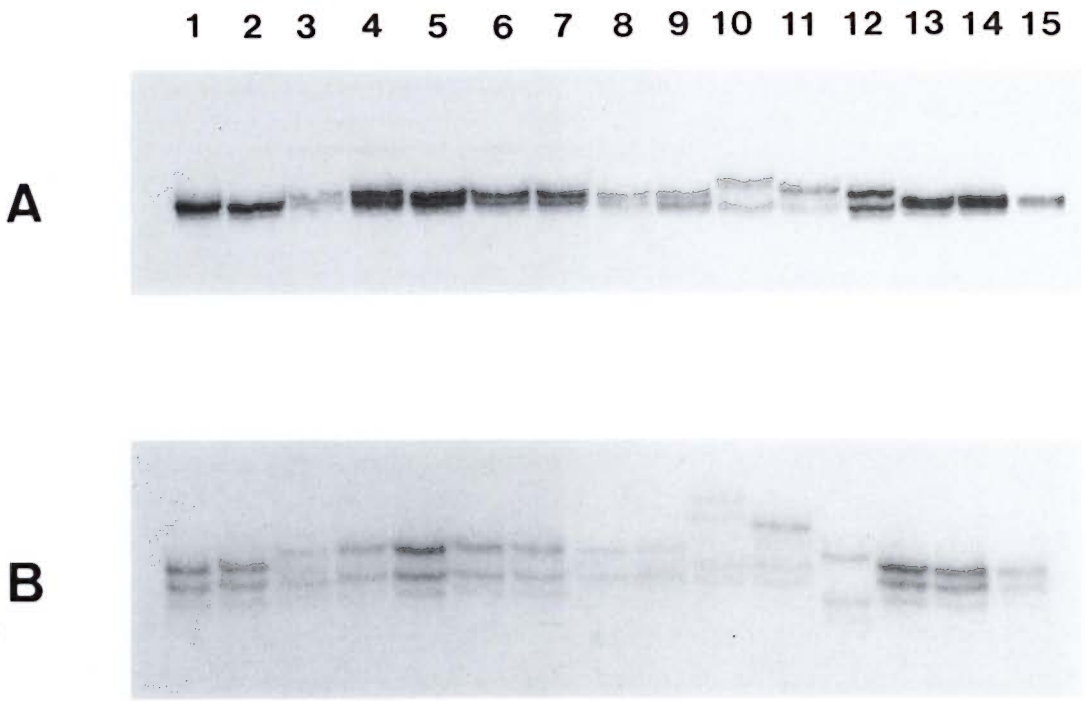

FIG. 2. **A.** SSCP analysis of the 390 bp PCR fragment obtained from the *rpoB* genes of various rifampin-sensitive (S) and -resistant (R) *M. leprae* strains. Lane 1, 88056 (S); lane 2, 90049 (S); lane 3, 82007 (R); lane 4, 85054 (R); lane 5, 86030 (R); lane 6, 82061 (R); lane 7, 86030 (R); lane 8, 83004 (R); lane 9, 82073 (R); lane 10 83013 (R); lane 11, 87038 (R); lane 12, 92041 (R); lane 13, 92002 (S); lane 14, 88063 (S); lane 15, 89033 (S). **B.** SSCP analysis of the 249 bp fragment obtained on *Pvu*I digestion of the 390 bp PCR fragment obtained from the *rpoB* genes of various *M. leprae* strains. Samples are as described in Figure 2A.

biopsy of a previously treated patient (92041; The Table), who had relapsed after rifampin therapy, was included in the PCR-SSCP analysis. Initially, the migration of the strands from the 390 bp PCR fragment obtained from the *rpoB* gene of 15 different strains was examined by electrophoresis. Although the differences in the mobilities of the strands were suboptimal, striking differences were apparent (Fig. 2). All of the rifampin-sensitive strains displayed the same electrophoretic pattern; the electrophoretic patterns of the mutants differed significantly. The mobilities of the strands from strains harboring the same mutations were identical but those from the other mutants, including the bacilli from the biopsy from patient 92041, displayed another pattern (Fig. 2A).

To increase the resolution of the technique, the size of the PCR fragment was reduced by cleavage with *Pvu*I. After SSCP-electrophoresis, the differences in the mobilities of the strands were much more obvious (Fig. 2B). Since the electrophoretic pattern obtained with the bacteria isolated from patient 92041 differed from that of the drug-sensitive strains this suggested that its

THE TABLE. *Properties of the M. leprae* isolates used in this study.

Strain	Allele	Rifampin[a]	Origin
82007	*rpoB1*	R	Martinque
85054	*rpoB2*	R	Paris (Martinique)
81030	*rpoB3*	R	Paris (Guadeloupe)
82061	*rpoB4*	R	New Caledonia
86030	*rpoB5*	R	Guadeloupe
83004	*rpoB6*	R	Martinique
82073	*rpoB7*	R	Paris
83013	*rpoB8*	R	Martinique
87038	*rpoB9*	R	Martinique
92041	*rpoB10*	R	Paris (Martinique)
88056	*rpoB*$^+$	S	Guadeloupe
90049	*rpoB*$^+$	S	New Caledonia
92002	*rpoB*$^+$	S	Martinique
88063	*rpoB*$^+$	S	Senegal
89033	*rpoB*$^+$	S	Paris

[a]R = resistant; S = susceptible.

rpoB gene, allele *rpoB10*, was indeed mutated. Furthermore, it also differed from all of the other mutants, thus indicating that a new type of mutation was present. To confirm this hypothesis the PCR fragment was subjected to DNA sequence analysis (data not shown). A single base change, C to G (Fig. 1A) from the wild type sequence ([6, 7]), was found in the 200 bp segment examined and this would lead to the replacement of His-520 by Asp in the β-subunit of RNA polymerase (Fig. 1B).

DISCUSSION

SSCP analysis is an extremely powerful technique for detecting mutations in short DNA fragments, and has found great application in the field of human genetic diseases such as cystic fibrosis or phenylketonuria ([1, 10]). In the present study, we have shown that it is also a most useful tool for screening for rifampin resistance in *M. leprae*. Initially, the method was optimized by using a panel of well-characterized resistant and susceptible strains, and striking differences in the electrophoretic mobilities of the strands of wild-type and mutant alleles of *rpoB* were seen. In this work PCR fragments were labeled with ^{32}P for reasons of ease. For routine application several alternatives are available, including the use of fluorescently tagged primers, incorporation of easily detectable modified bases, such as digitonin-labeled dUTP, or silver staining of unlabeled DNA ([1, 9]). Further improvements could be obtained by using primers, flanking the region of interest, that give rise to a smaller PCR product, thus obviating the need for the *Pvu*I digestion.

To test the predictive powers of SSCP, bacilli suspected of being rifampin resistant, from a skin biopsy of patient 92041, were analyzed and a novel electrophoretic pattern was found. Subsequent DNA sequence studies revealed a C-to-G transversion in codon 420 of *rpoB* which resulted in the substitution of a histidine residue by aspartic acid. Exactly the same mutation has been found in rifampin-resistant isolates of *M. tuberculosis* (Fig. 1) ([8, 13]), thus indicating that bacilli from biopsy 92401 almost certainly are resistant. To confirm this, their drug susceptibility is currently being assessed in mice although the results will not be available for 1 year. This point underlines the great value of a combined PCR-SSCP approach because one can perform the reactions directly on a small biopsy sample, containing 100–1000 *M. leprae* cells, and obtain the results within 48 hr. This means that in cases of suspected relapse, or resistance, insight can be obtained rapidly thus allowing appropriate action, such as a change of drug regimen, to be taken without delay.

SUMMARY

The rifampin resistance of *Mycobacterium leprae* is due to missense mutations in the *rpoB* gene encoding the β-subunit of the essential enzyme RNA polymerase. A rapid and very simple method has been developed to detect rifampin resistance in small numbers of *M. leprae* present in biopsies. It involves polymerase chain reaction amplification of a defined region of the *rpoB* gene followed by single-strand conformational polymorphism analysis (PCR-SSCP). The reliability of the method has been tested on a sample of known drug-resistant and -susceptible isolates of *M. leprae*.

RESUMEN

La resistencia del *Mycobacterium leprae* a la rifampina se debe a mutaciones sin sentido en el gene *rpoB* que codifica la subunidad β de la enzima esencial RNA-polimerasa. Nosotros desarrollamos un método rápido y muy simple para detectar la resistencia a rifampina en pequeños números de *M. leprae* presentes en biopsias. El método involucra la amplificación por la reacción en cadena de la polimerasa de una región definida del gene *rpoB*, seguido por un análisis del polimorfismo conformacional del DNA de cadena simple (PCR-SSCP). La confiabilidad del método se ha probado usando especímenes de *M. leprae* susceptibles y resistentes a la droga.

RÉSUMÉ

La résistance à la rifampicine de *Mycobacterium leprae* est dûe à des mutations désordonnées dans le gène rpoB encodant la sub-unité β de l'enzyme essentiel ARN-Polymerase. Une méthode rapide et très simple at été développée pour détecter la résistance à la rifampicine sur les faibles quantiés de *M. leprae* présentes dans les biopsies. Elle inclut la réaction d'amplification de la polymerase en chaîne d'une région précise du gène rpoB suivie d'une analyse polymorphique de la conformation d'un brin momocaténaire (PCR-SSCP). La fiabilité de la méthode a été testée sur un échantillon d'isolates de *M. leprae* dont la résistance ou la sensibilité à la rifampicine était connue.

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