

Detection of Rifampin Resistance by Single-Strand Conformation Polymorphism Analysis of Cerebrospinal Fluid of Patients with Tuberculosis of the Central Nervous System

PAOLO SCARPELLINI,^{1*} SERGIO BRAGLIA,¹ ANNA MARIA BRAMBILLA,¹
MARGHERITA DALESSANDRO,¹ PAOLA CICHERO,² ANDREA GORI,³ AND ADRIANO LAZZARIN¹

*Infectious Diseases Division¹ and Laboratory of Microbiology,² San Raffaele Scientific Institute, and
Clinic of Infectious Diseases, University of Milan,³ Milan, Italy*

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Mutations in a 69-bp region of the *rpoB* gene of *Mycobacterium tuberculosis* are associated with rifampin resistance (Rif^r). These have been detected with mycobacterial DNA extracted from bacterial suspensions or respiratory specimens that were acid-fast smear positive. We experimented with a strategy for the rapid detection of Rif^r in cerebrospinal fluid (CSF) samples. The strategy involves the amplification of the 69-bp region of *rpoB* by means of PCR and the identification of nucleotide mutations by single-strand conformation polymorphism (SSCP) analysis of the amplification products. Sixty-five CSF specimens collected from 29 patients (19 patients were coinfecting with human immunodeficiency virus) with culture or autopsy-confirmed (22 patients) or highly probable (7 patients) tuberculosis of the central nervous system (CNS-TB) were processed. Amplified products suitable for evaluation by SSCP analysis were obtained from 37 CSF specimens from 25 subjects (86.2%). PCR-SSCP of CSF correctly identified the rifampin susceptibility phenotype of isolates from all 17 patients for whom the results of susceptibility tests carried out with strains cultured from CSF or respiratory samples were available. Moreover, this assay revealed the rifampin susceptibility genotype of isolates from the eight patients (three patients with culture-confirmed CNS-TB and five patients in whom CNS-TB was highly probable) for whom no susceptibility test results were available; the PCR-SSCP data obtained for these patients were concordant with the outcome after a standard antituberculosis treatment. The evolution of a mutation in the *rpoB* gene was documented in a patient during the course of treatment. PCR-SSCP analysis of CSF seems to be an efficacious method of predicting Rif^r and would reduce the time required for susceptibility testing from approximately 4 to 8 weeks to a few days.

Tuberculosis is still an important cause of morbidity and mortality throughout the world. The steady decline in the incidence of tuberculosis, both in the Third World and in industrialized countries, which followed the introduction of effective chemotherapy, was reversed in the mid-1980s mainly due to epidemic human immunodeficiency virus (HIV) infection (6, 14). Tuberculosis of the central nervous system (CNS-TB) is a severe condition which, if left untreated, leads to a fatal outcome within 4 to 8 weeks of the onset of symptoms. An early and accurate diagnosis and administration of an effective chemotherapy could improve the outcome and reduce the neurological sequelae (13, 17). The increased occurrence of tuberculosis is paralleled by the emergence of multidrug-resistant isolates of *Mycobacterium tuberculosis*, which are at least resistant to rifampin and isoniazid (5). The rapid availability of information concerning drug susceptibility patterns is important not only because it makes it possible to select adequate multidrug regimens but also because it contributes to minimizing the spread of drug-resistant strains. Since isolation, identification, and susceptibility testing by conventional procedures can take 4 to 8 weeks, it would be a great advantage to be able to obtain accurate results in a shorter time.

Rifampin is an essential component of therapeutic regimens and is most frequently used in combination with isoniazid,

pyrazinamide, and ethambutol. Rifampin resistance (Rif^r) in mycobacteria is most often due to point mutations and small insertions and deletions located in a 69-bp region of the *rpoB* gene encoding the β -subunit of the RNA polymerase (4, 7, 10, 12, 19, 20, 22, 23), a mechanism that was first documented in *Escherichia coli* and *Mycobacterium leprae* (9, 11). DNA-based diagnostic assays, such as single-strand conformation polymorphism (SSCP), heteroduplex formation, and automated DNA sequence analyses have been used for the detection of mutations in the PCR amplification products of the *rpoB* gene (4, 7, 10, 12, 19, 20, 22, 23), but the detection of Rif^r by these methods has largely been limited to bacterial suspensions of clinical isolates of *M. tuberculosis* or clinical specimens with a high burden of bacilli, such as smear-positive respiratory samples.

We present here the results of our experience with a PCR assay coupled with a manual SSCP analysis for the evaluation of Rif^r directly from the cerebrospinal fluid (CSF) of patients with confirmed or highly probable CNS-TB. The PCR-SSCP analysis data obtained by processing both CSF samples and *M. tuberculosis* strains isolated from the same patients were compared with the rifampin susceptibility phenotype. Sequence analysis of PCR products from bacterial suspensions was carried out in order to characterize the mutations. The sensitivity of PCR amplification of the *rpoB* sequence was compared with that of another homemade nested PCR protocol amplifying IS6110. The clinical strains were also characterized by examining their IS6110 restriction fragment length polymorphism (RFLP) patterns in order to determine the spread of a limited number of clones.

* Corresponding author. Mailing address: Infectious Diseases Division, San Raffaele Scientific Institute, Via Stamira D'Ancona 20, 20127 Milan, Italy. Phone: 0039-2-2643-7939. Fax: 0039-2-2643-7030. E-mail: scarpep@hsr.it.

MATERIALS AND METHODS

Clinical samples. We studied a total of 65 CSF samples obtained by means of diagnostic lumbar punctures from 29 patients (19 patients were HIV seropositive) admitted to various hospitals in northern Italy over a period of 6 years (1992 to 1997). Only a single CSF sample was available from 11 patients, whereas additional specimens were also collected from the remaining 18 patients during the administration of antituberculosis compounds (see Table 1). The clinical diagnoses of CNS-TB were confirmed by means of *M. tuberculosis*-positive CSF cultures for 19 patients and at autopsy for 3 patients; for the remaining 7 patients, the diagnosis of CNS-TB was considered highly probable because the suggestive clinical picture was accompanied by at least three of the following supporting tests: (i) compatible abnormal CSF findings, including increased leukocyte counts (mainly lymphocytes), hypoglycorrhachia, protein concentration of more than 100 mg/dl, and sterile routine bacterial and fungal cultures; (ii) computed tomography findings suggesting CNS-TB (basal exudate, hydrocephalus, and intracranial tuberculomas); (iii) evidence of tuberculosis outside the CNS; and (iv) clinical response to antituberculosis therapy (Table 1).

An aliquot of each CSF sample underwent microscopic examination for acid-fast bacilli, culture for *M. tuberculosis*, and DNA amplification by two PCR protocols. The first PCR protocol amplified a fragment of IS6110, and the second produced an amplification product of the region identified as the Rif^r locus contained in the *rpoB* gene, which was used for the subsequent detection of mutations by SSCP analysis. CSF aliquots of 1 to 2 ml were concentrated by centrifugation (3,000 × g for 10 min) for conventional bacteriological examination; the sediments were used to prepare smears for direct examination by means of auramine-rhodamine staining and were cultured by inoculation on Löwenstein-Jensen medium (samples processed before 1993) or BACTEC 12B medium (Becton Dickinson Diagnostic Instruments, Sparks, Md.) for radiometric detection of mycobacterial growth (samples processed from 1993 onward). The cultures on solid media were aerobically incubated in an atmosphere with 5% CO₂ at 37°C. All cultures were observed for 12 weeks before they were discarded. Routine biochemical methods and the Accuprobe culture confirmation kits (Gen-Probe, San Diego, Calif.) were used to identify the isolates. The susceptibility to rifampin was determined by the BACTEC radiometric method. The critical concentration of rifampin in BACTEC 12B vials was 2 µg/ml. The MICs for resistant strains were measured by the dilution method on Middlebrook 7H11 agar medium (8).

Mycobacterial strains. Seventeen *M. tuberculosis* strains cultured from patients with CNS-TB were used for PCR-SSCP analysis and for direct DNA sequencing. Strains were classified on the basis of IS6110 RFLP pattern subtypes, according to internationally standardized guidelines (21). The rifampin-sensitive isolate *M. tuberculosis* H37rv (ATCC 27294) and seven rifampin-resistant *M. tuberculosis* clinical isolates with different mutations located in the Rif^r region were used as controls.

Sample preparation and PCR amplification. Bacterial suspensions, containing approximately 10⁵ acid-fast bacilli in 250 µl of sterile deionized H₂O and 500 µl of each CSF specimen underwent DNA extraction and purification by the chaotropic-silica method described by Boom et al. (3). The DNA (10 µl) extracted from the bacterial suspensions was used for amplification of the Rif^r region (GenBank accession no. L05910) with primers TR9 (5'-TCGCCGCGATC AAGGAGT) and TR8 (5'-TGCACGTCGCGGACCTCCA) by the protocol described by Telenti et al. (20). The DNA (10 µl) extracted from the CSF specimens was used for time-release PCR of the Rif^r region with primers TR8 and TR9. The PCR mixture (100 µl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 200 µM (each) dATP, dGTP, and dTTP, 100 µM dCTP, 0.5 µl (5 µCi) of [³²P]dCTP, and the primers (0.5 µM each); 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.) was added to each tube. The reaction was subjected to a 2-min pre-PCR heating step at 95°C, followed by 20 PCR cycles of 1 min each at 95, 63, and 72°C, 30 PCR cycles of 30 s at 96°C and 1 min each at 60 and 72°C, and a final 15-min extension at 72°C in a Perkin-Elmer 480 thermal cycler. In order to analyze the PCR product, 10 µl of the reaction solution was electrophoresed on a 1.8% agarose gel containing 0.5 µg of ethidium bromide per ml. The presence of a 157-bp band indicated a successful amplification. To exclude cross-contamination, a negative control containing all the ingredients of the PCR mixture except the DNA template was included for every two samples. Precautions to avoid false-positive results were taken in every assay, as described previously (16).

Each CSF sample was also subjected to a nested PCR protocol for the final amplification of a 123-bp product contained in IS6110, as described previously (18).

The sensitivities of the two PCR protocols were determined by testing serial twofold dilutions of an *M. tuberculosis* clinical strain as described by Kolk et al. (15). The number of CFU per milliliter was estimated by means of routine plating and colony counting by using triplicate Middlebrook 7H10 agar plates. Simple heating to 95°C for 15 min was used to release DNA from bacterial cells prior to amplification.

SSCP analysis. SSCP analysis was performed by mixing 25 µl of the PCR product with 100 µl of SSCP dilution solution (10 mM EDTA, 0.1% sodium dodecyl sulfate). The diluted product (10 µl) was mixed with 10 µl of gel loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA), and the mixture was heated for 10 min at 98°C, cooled on ice for 10 min,

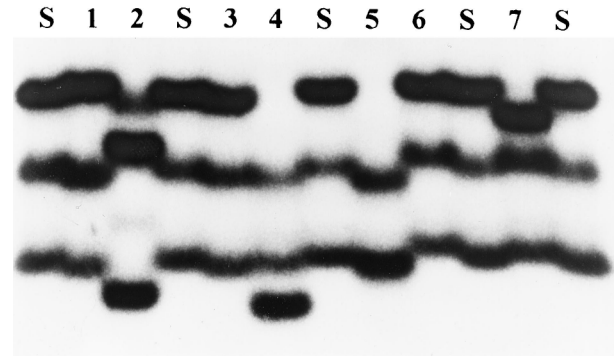


FIG. 1. PCR-SSCP patterns obtained by processing rifampin-resistant *M. tuberculosis* clinical isolates (samples [lanes] 1 to 7) and a rifampin-sensitive *M. tuberculosis* reference strain (ATCC 27294) (lanes S). The rifampin-resistant strains contained mutations in the *rpoB* gene located in codon 516, reading GTC instead of GAC (Val→Asp) (sample 1); codon 526, reading AAC, CTC, GAC, or TAC instead of CAC (His→Asn, Leu, Asp, or Tyr) (samples 2 to 5); codon 531, reading TTG instead of TCG (Ser→Leu) (sample 6); and codon 533, reading CCG instead of CTG (Leu→Pro) (sample 7). All of these kinds of single point substitutions were reported previously (12, 20). The finding of a three-band SSCP pattern may reflect the presence of two possible conformations for one of the DNA strands or a reannealing band. The codon numbers correspond to *E. coli* RNA polymerase amino acid positions (11).

and loaded onto a nondenaturing sequencing format 0.5× MDE gel composed of 10 ml of MDE (Hydrolink; AT Biochem Inc., Malvern, Pa.), 4.8 ml of 5× TBE buffer (0.45 M Tris-borate, 10 mM EDTA), and 25.2 ml of H₂O polymerized with 40 µl of 25% ammonium persulfate and 50 µl of *N,N,N',N'*-tetramethylethylenediamine (TEMED; Bio-Rad, Richmond, Calif.). Electrophoresis was performed at room temperature overnight at constant power (6 W for a gel of 55 by 20 by 0.04 cm). The gels were dried for 1 h at 80°C and were exposed overnight for autoradiography.

DNA sequencing. Purified *M. tuberculosis* DNA from the clinical isolates and reference strain was used to produce a 350-bp fragment of *rpoB* by using the primers and PCR conditions described by Kapur et al. (12). The unincorporated nucleotides and primers were separated from the amplified DNA using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing was carried out with an ABI 373 automated sequencer by using the PRISM dye terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions. Both strands were sequenced. The data were assembled and edited by using the Sequencer, version 3.0, analysis program (Gene Codes Corporation).

RESULTS

Sensitivities of PCR protocols. The detection limits of the PCR for the *rpoB* gene and IS6110 of *M. tuberculosis* were 100 and 50 CFU/ml of the bacterial suspensions, respectively.

SSCP analysis. Figure 1 shows the SSCP patterns obtained by processing the rifampin-sensitive strain *M. tuberculosis* H37rv (ATCC 27294) and seven rifampin-resistant *M. tuberculosis* clinical isolates with different mutations located in the Rif^r region that were used as controls.

Table 1 summarizes the results obtained by processing 65 CSF samples from 29 patients suffering from CNS-TB by using the two PCR protocols, SSCP analysis of the amplified products of *rpoB*, and testing of the susceptibilities of the clinical isolates to rifampin. A positive result was obtained for at least one CSF sample from each patient with CNS-TB by the IS6110 PCR. Amplified products suitable for subsequent SSCP analysis were obtained from 37 CSF samples drawn from 25 of the 29 patients (86.2%) by the *rpoB* PCR. SSCP analysis showed single DNA strands with electrophoretic mobilities that were identical to that of a rifampin-sensitive wild-type strain (*M. tuberculosis* H37rv [ATCC 27294]) for *rpoB* PCR products from 19 samples collected from 17 patients with confirmed or highly probable cases of CNS-TB, respectively (Table 1). Twelve *M. tuberculosis* isolates were cultured from the CSF

TABLE 1. SSCP analysis of CSF samples, patient data, results obtained by two PCR protocols, patterns by PCR-SSCP and susceptibilities of clinical isolates to rifampin

Infection status and patient no.	Age (yr)	Sex ^a	No. of CSF samples			Pattern by PCR-SSCP analysis of CSF ^b	Rifampin phenotype
			Total	Positive by IS6110 PCR	Positive by <i>rpoB</i> PCR (evaluable by SSCP)		
Confirmed to have CNS-TB by CSF-positive culture ^c							
1	32	M ^d	5	3	1	Wild type	Sensitive
2	27	F ^d	3	2	1	Wild type	Sensitive
3	72	M	1	1	1	Wild type	Sensitive
4	83	M ^d	3	2	1	Wild type	Not available
5	30	M	1	1	1	Wild type	Sensitive
6	42	F ^d	4	3	3	Wild type	Sensitive
7	30	M ^d	1	1	1	Wild type	Not available
8	56	M ^d	2	1	1	Wild type	Sensitive
9	80	F	2	2	1	Wild type	Not available
10	31	F	3	2	1	Wild type	Sensitive
11	40	M ^d	1	1	1	Wild type	Sensitive
12	65	M	1	1	1	Wild type	Sensitive
13	40	F	2	1			Sensitive
14	40	M ^d	2	1			Sensitive
15	31	M ^d	1	1			Sensitive
16	34	M ^d	1	1	1	Abnormal	Resistant
17	35	M ^d	1	1	1	Abnormal	Resistant
18	30	M ^d	4	3	1	Abnormal	Resistant
19	26	M ^d	3	3	3	Abnormal	Resistant
Confirmed to have CNS-TB at autopsy							
20	30	M	1	1	1	Abnormal	Resistant ^e
21	33	M ^d	4	4	4	Abnormal	Resistant ^e
22	36	M ^d	2	2	2	Abnormal	Resistant ^e
Highly probable cases							
23	51	F	2	1	1	Wild type	Not available
24	27	F ^d	2	2	1	Wild type	Not available
25	29	M ^d	2	1	1	Wild type	Not available
26	30	M ^d	1	1	1	Wild type	Not available
27	29	M	1	1			Not available
28	30	F ^d	2	2	2	Abnormal	Resistant ^e
29	41	F	7	3	4	Wild type and Abnormal ^f	Not available
Total no. of samples (no. of patients)			65 (29)	49 (29)	37 (25)		

^a M, male; F, female.^b SSCP patterns were classified as wild type or abnormal in the case of single DNA strands with electrophoretic mobilities identical to or different from that of a reference strain (*M. tuberculosis* H37rv [ATCC 27294]), respectively.^c See Materials and Methods for more information on classification criteria.^d Patients coinfecting with HIV.^e Susceptibility testing determined with isolates cultured from respiratory specimens.^f SSCP analysis showed a wild type in the baseline and an abnormal pattern in three subsequent CSF samples.

specimens from these patients. Testing of susceptibility to rifampin was carried out for nine of these strains and revealed a sensitive phenotype. SSCP analysis showed an electrophoretic mobility that was clearly different from that of the reference strain for the *rpoB* PCR products obtained from 18 samples drawn from nine patients with confirmed or highly probable CNS-TB (Table 1). Among these patients three different SSCP patterns were identified (Fig. 2). Eight *M. tuberculosis* strains were cultured from the CSF samples or respiratory specimens collected from these patients. All of these strains had phenotypes of rifampin resistance, and the MICs for these strains were ≥ 128 mg/liter. From patient 29 (Table 1; Fig. 2), we processed a total of seven CSF samples collected over a 2-year period. This patient had severe meningoencephalitis in 1994, with the partial resolution of the symptoms and the neurora-

biological picture after 6 months of antituberculosis therapy; then the patient worsened again and the therapy was modified. SSCP analysis showed a change from a wild-type to an abnormal pattern from the baseline to three subsequent CSF samples collected during the two episodes. The CSF samples collected from this patient were negative upon culture and microscopic examination.

The SSCP patterns obtained by processing the amplified products obtained from suspensions of 17 *M. tuberculosis* isolates and from CSF specimens collected from the same patients were also compared; no difference in the SSCP patterns was observed.

Direct DNA sequencing and RFLP analysis. Seven different kinds of point mutation were revealed in the rifampin-resistant *M. tuberculosis* clinical strains that we used as controls. For

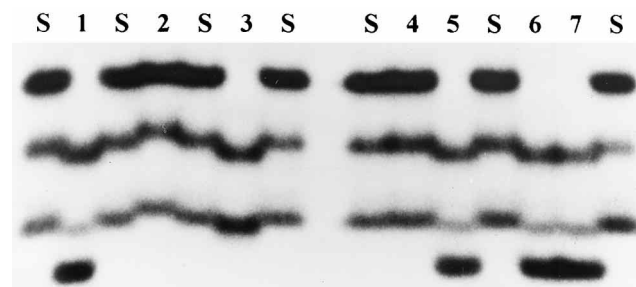


FIG. 2. PCR-SSCP patterns obtained by processing CSF samples from patients with CNS-TB. Lanes S, pattern obtained from rifampin-sensitive *M. tuberculosis* reference strain (ATCC 27294). Samples (lanes) 1 to 3, patterns obtained from specimens collected from patients with CNS-TB caused by rifampin-resistant strains. These patterns were expressions of single base substitutions in codon 526 of the *M. tuberculosis rpoB* gene, reading GAC instead of CAC (His→Asp); in codon 531, reading TTG instead of TCG (Ser→Leu); and in codon 526, reading TAC instead of CAC (His→Tyr), respectively. Samples (lanes) 4 to 7, patterns obtained from specimens drawn from a patient (patient 29; see Table 1) before and during antituberculosis treatment; note the change from a wild-type pattern (sample 4) to an abnormal one (samples 5 to 7), which may have been an expression of selection of a clone carrying a mutation which causes a Rif^r phenotype. This finding correlates well with the clinical and neuroradiological evolution of the disease in this patient.

each nucleotide substitution a specific and characteristic SSCP pattern was observed (Fig. 1). No mutations were revealed in the *rpoB* segment sequenced from the rifampin-sensitive reference strain (ATCC 27294).

Seventeen *M. tuberculosis* strains (nine rifampin-sensitive and eight rifampin-resistant strains) cultured from samples collected from patients with CNS-TB were available for direct DNA sequencing. No mutations were revealed in the *rpoB* segment sequenced from the rifampin-sensitive strains, whereas single point mutations were detected in all of the strains with a resistant phenotype. Three different kinds of nucleotide substitution were revealed in two codons of the *rpoB* gene, which were specific and characteristic for each SSCP pattern (Fig. 2). The mutations were located in codon 531, reading TTG instead of TCG (Ser→Leu), and in codon 526, reading GAC and TAC instead of CAC (His→Asp and His→Tyr). These three single-base substitutions represent 56 to 68% of the mutations associated with rifampin resistance detected worldwide (4, 12, 20, 23).

The 17 *M. tuberculosis* strains were also analyzed by means of RFLP analysis with IS6110 as a hybridization probe. The number of IS6110 copies in these strains varied from 1 to 14, and a total of nine and four RFLP patterns were identified for susceptible and resistant strains, respectively. Patients 17 to 22 suffered from disseminated tuberculosis sustained by two distinct multidrug-resistant isolates that caused two nosocomial outbreak in four infectious diseases divisions in northern Italy (data not shown).

DISCUSSION

The presence of mutations in a restricted sequence of the *rpoB* gene has been found in more than 96% of *M. tuberculosis* strains with various levels of Rif^r (4, 7, 10, 12, 19, 20, 22, 23); the degree of resistance, determined by measuring the MICs, seems to be related to the type of mutation (2). The use of molecular biology-based techniques in the search for Rif^r at the genomic level is more rapid (and therefore of greater help in therapeutic decision making) than the use of conventional methods based on verifying the growth of the organism in antibiotic-containing culture media.

Various investigators have reported encouraging results concerning the validity of this new approach when the DNA is extracted from mycobacterial cultures (4, 7, 10, 12, 19, 20, 22, 23), but the detection of mutations in DNA extracted directly from pathological clinical samples (which would save an even greater amount of time) is still hampered by unresolved methodological problems mainly due to the poor sensitivity and specificity of the adopted amplification protocols (10, 23). Because only a single copy of the *rpoB* gene is present in mycobacterial DNA, PCR protocols with this target are less sensitive than those amplifying repeated sequences, such as IS6110. Furthermore, given that the *rpoB* gene is highly conserved in the various GC-rich bacterial species colonizing the respiratory tract, it is possible that the generation of nonspecific amplification products may interfere with subsequent analysis (10). It is for this reason that, at least in the early studies, the detection of Rif^r at the genomic level by means of SSCP, heteroduplex formation, and DNA sequencing analyses of PCR products was only possible with samples with a high mycobacterial load that could be revealed by microscopy (10, 11). Whelen et al. (22) have recently optimized a single-tube heminested PCR protocol and obtained *rpoB* gene amplification products for 87.5% of various types of culture-positive samples, some of which were negative by microscopic examination. Nevertheless, the use of highly sensitive double amplification protocols not only increases the risk of contamination but could theoretically lead to the generation of amplification products from only a few DNA targets, which means that there is a risk that the subsequent analysis of the amplified DNA may not reflect the real percentage of mutated organisms among the bacterial population in the pathological sample.

The present paper reports the results of the SSCP and DNA sequencing analyses of *rpoB* gene amplification products obtained by processing CSF samples and *M. tuberculosis* strains from subjects with CNS-TB. Amplification products suitable for the subsequent detection of mutations were obtained from the CSF samples drawn from 16 (84.2%) of the 19 subjects whose CNS-TB was confirmed by means of cultural investigations and from 9 (90%) of the 10 subjects whose CSF cultures were negative but whose disease was confirmed at autopsy or considered highly probable. Overall, SSCP analysis proved to be possible for 86.2% of the patients. This result was achieved thanks to the use of the mycobacterial DNA concentration, extraction, and purification process applied to large volumes of CSF and the use of a highly specific enzyme that can be activated at high temperatures, which made it possible to increase the number of amplification cycles (1). The sensitivity of this method was similar to that of a nested PCR protocol based on the final synthesis of the same sequence and use of the outer primers described by Kapur et al. (12) (data not shown).

In our experience, the detection of rifampin susceptibility by means of the genotypic study of the mycobacterial DNA present in pathological samples, using SSCP analysis as an indirect method of revealing the presence of mutations, has proved to be an approach that is highly predictive of the strain's phenotype determined by conventional susceptibility testing. All of the *M. tuberculosis* strains cultured from the CSF or respiratory samples obtained from 17 patients with CNS-TB showed a rifampin phenotype that was in line with the results of the SSCP analysis. For eight patients, it was not possible to compare the rifampin phenotype with the SSCP data because no susceptibility testing results were available. The clinical and radiological documentation concerning these patients was critically reviewed with the aim of evaluating the evolution of the disease after the initiation of specific treatment with isoniazid, rifampin, ethambutol, and pyrazinamide. No evaluation was

possible for three patients (patients 7, 23, and 24), because they died within a week of the onset of the disease, whereas a complete cure was obtained for four patients (patients 4, 9, 25, and 26) (data not shown). SSCP analysis did not reveal any *rpoB* gene mutations in the genomes of the mycobacterial isolates from the CSF of these patients. Analysis of the amplified products obtained by processing four samples drawn from patient 29 before and during antibiotic treatment revealed a change from a wild-type to an altered SSCP pattern, which may have been an expression of a mutation which causes a Rif^r phenotype. This finding correlates well with the clinical and neurological evolution of the disease in this subject: The first 6 months of treatment led to an encouraging improvement, as demonstrated by the partial regression of some encephalitic foci, but the disease gradually worsened again until the onset of hydrocephalus and the need for drainage.

In conclusion, SSCP analysis of *rpoB* gene amplification products obtained from mycobacterial DNA taken from the CSF of subjects with CNS-TB appears to be a promising method for determining mutations that confer resistance to rifampin. Its use can radically reduce the time needed to provide clinicians with data that are useful in aiding the selection of active drugs for the efficacious treatment of tuberculosis.

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REFERENCES

- Birch, D. E., L. Kolmodin, W. J. Laird, N. McKinney, J. Wong, K. K. J. Young, G. A. Zangenberg, and M. A. Zoccoli. 1996. Simplified hot start PCR. *Nature* **381**:445–446.
- Bodmer, T., G. Zurcher, P. Imboden, and A. Telenti. 1995. Mutation position and type of substitution in the β -subunit of the RNA polymerase influence in-vitro activity of rifamycins in rifampicin-resistant *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* **35**:345–348.
- Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**:495–503.
- Caugant, D. A., P. Sandven, J. Eng, J. T. Jeque, and T. Tojum. 1995. Detection of rifampin resistance among isolates of *Mycobacterium tuberculosis* from Mozambique. *Microb. Drug Resist.* **1**:321–326.
- Centers for Disease Control and Prevention. 1993. Initial therapy for tuberculosis in the era of multidrug resistance: recommendations of the Advisory Council for the Elimination of Tuberculosis. *Morbid. Mortal. Weekly Rep.* **42**:1–8.
- Dolin, P. J., M. C. Raviglione, and A. Kochi. 1994. Global tuberculosis incidence and mortality during 1990–2000. *Bull. W. H. O.* **72**:213–220.
- Felmlee, T. A., Q. Liu, A. C. Whelen, D. Williams, S. S. Sommer, and D. H. Persing. 1995. Genotypic detection of *Mycobacterium tuberculosis* rifampin resistance: comparison of single-strand conformation polymorphism and dideoxy fingerprinting. *J. Clin. Microbiol.* **33**:1617–1623.
- Heifets, L. B. 1991. Antituberculous drugs: antimicrobial activity in vitro, p. 14–57. In L. B. Heifets (ed.), *Drug susceptibility in the chemotherapy of mycobacterial infections*, 1st ed. CRC Press, Inc., Boca Raton, Fla.
- Honoré, N., and S. Cole. 1993. The molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob. Agents Chemother.* **37**:414–418.
- Hunt, J. M., G. D. Roberts, L. Stockman, T. A. Felmlee, and D. H. Persing. 1994. Detection of a genetic locus encoding resistance to rifampin in mycobacterial cultures and in clinical specimens. *Diagn. Microbiol. Infect. Dis.* **18**:219–227.
- Jin, D., and C. A. Gross. 1988. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J. Mol. Biol.* **202**:45–58.
- Kapur, V., L.-L. Li, S. Iordanescu, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser. 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase β subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *J. Clin. Microbiol.* **32**:1095–1098.
- Kennedy, D. H., and R. J. Fallon. 1979. Tuberculous meningitis. *JAMA* **241**:264–268.
- Kochi, A. 1994. Global challenge of tuberculosis. *Lancet* **344**:608–609.
- Kolk, A. H. G., A. R. J. Schuitema, S. Kuijper, J. van Leeuwen, P. W. M. Hermans, J. D. A. van Embden, and R. A. Hartskeerl. 1992. Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a non radioactive detection system. *J. Clin. Microbiol.* **10**:2567–2575.
- Kwok, S., and R. Higuichi. 1989. Avoiding false positives with PCR. *Nature* **339**:237–238.
- Leonard, D. J., and R. M. Des Prez. 1990. Tuberculous meningitis. *Infect. Dis. Clin. N. Am.* **4**:769–787.
- Scarpellini, P., S. Racca, P. Cinque, F. Delfanti, N. Gianotti, M. R. Terreni, L. Vago, and A. Lazzarin. 1995. Nested polymerase chain reaction for diagnosis and monitoring treatment response in AIDS patients with tuberculous meningitis. *AIDS* **9**:895–900.
- Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer. 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* **341**:647–650.
- Telenti, A., P. Imboden, F. Marchesi, T. Schmidheini, and T. Bodmer. 1993. Direct, automated detection of rifampin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. *Antimicrob. Agents Chemother.* **37**:2054–2058.
- van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol.* **31**:406–409.
- Whelen, A. C., T. A. Felmlee, J. M. Hunt, D. L. Williams, G. D. Roberts, L. Stockman, and D. H. Persing. 1995. Direct genotypic detection of *Mycobacterium tuberculosis* rifampin resistance in clinical specimens by using a single tube heminested PCR assay. *J. Clin. Microbiol.* **33**:556–561.
- Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M. Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis. 1994. Characterization of rifampin resistance in pathogenic mycobacteria. *Antimicrob. Agents Chemother.* **38**:2380–2386.