

# Detection of rifampin resistant tuberculosis meningitis by polymerase chain reaction linked single strand conformation polymorphism analysis of cerebrospinal fluid

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## ABSTRACT

**Objective:** To rapidly detect rifampin resistance in *Mycobacterium tuberculosis* isolates causing meningitis in northeast Iran.

**Methods:** This study presents the results of a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis for the evaluation of rifampin resistance directly from the CSF of 47 patients strongly suspicious to have tuberculosis meningitis in Emam Reza University Hospital, Mashhad, Iran over 3 years (2002 to 2005). Each CSF sample underwent microscopic examination, culture and DNA amplification by 2 PCR protocols and subsequent detection of mutations by SSCP analysis.

**Results:** Among these patients, no mutations were revealed in the *rpoB* segment by SSCP.

**Conclusion:** The SSCP analyses of these samples shows complete susceptibility to rifampin. The use of this method can radically reduce the time needed to provide clinicians with data useful in aiding the selection of appropriate drugs.

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Despite major advances in the prevention and treatment of tuberculosis in the past century, this disease remains a global threat to public health.<sup>1-3</sup> Tuberculosis meningitis is a

severe condition, which leads to a fatal outcome. An early and accurate diagnosis and administration of an effective chemotherapy could improve the outcome and reduce the neurological sequelae.<sup>4,5</sup> Rifampin is an essential component of therapeutic regimens, which is most frequently used in combination with isoniazid, pyrazinamide, and ethambutol. Consequently, resistance of *Mycobacterium tuberculosis* (*M. tuberculosis*) strains to rifampin limits the effectiveness of tuberculosis control programs by complicating both treatment and prevention protocols.<sup>6-10</sup> The rapid availability of information concerning drug susceptibility patterns is important because it makes it possible to select appropriate regimens and so minimizes the spread of drug-resistant strains. Since isolation, identification, and susceptibility testing by conventional procedures can take 4-8 weeks, it would be a great advantage to be able to obtain accurate results in a shorter time. Rifampin resistance in *M. tuberculosis* is most often due to mutations located in an 81-bp region of the *rpoB* gene encoding the  $\beta$ -subunit of the RNA polymerase.<sup>11-14</sup> The DNA-based diagnostic assays, such as single-strand conformation polymorphism (SSCP) have proved suitable methods for the detection of mutations in the polymerase chain reaction (PCR) amplification products of the *rpoB* gene.<sup>11-17</sup> We present here the results of our experience with a PCR assay coupled with a SSCP analysis for the evaluation of rifampin resistance directly from the CSF of patients with tuberculosis meningitis.

**Methods. Clinical samples.** We studied 47 CSF samples obtained by diagnostic lumbar punctures from patients strongly suspicious to have tuberculosis meningitis in Emam Reza University Hospital, Mashhad, Iran over a period of 3 years (2002 to 2005). Each CSF sample underwent microscopic examination for acid-fast bacilli, culture for *M. tuberculosis*, and DNA amplification by 2 PCR protocols. The first PCR protocol amplified a 123-bp fragment of IS6110,<sup>18</sup> and the second produced the region identified as the rifampin resistance locus (Rifr) contained in the *rpoB* gene, which was used for the subsequent detection of mutations by SSCP analysis. The diagnoses of tuberculosis

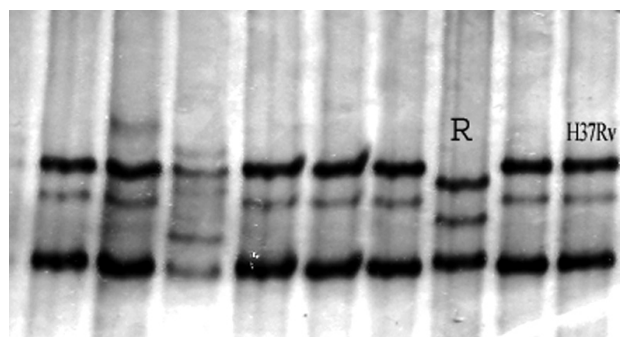
meningitis were confirmed by means of positive laboratory tests as direct microscopic examination, culture, or PCR for 24 patients.

**Sample preparation and PCR amplification.** From each CSF specimen, 500 µl underwent DNA extraction and purification.<sup>19</sup> The DNA (1 µl) extracted from the CSF specimens was used for amplification of the Rifr region (GenBank accession no. L05910) with primers Rs (5'-GGTCGCCGCGATCAAGG) and Ra (5'-CGACAGCGAGCCGATCAG).<sup>20</sup> The PCR mixture (20 µl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2 mM MgCl<sub>2</sub>, 200 mM dNTP, the primers (2 pmol each) and 1 unit of Taq DNA polymerase was added to each tube. The reaction was subjected to a 3 minute pre-PCR heating step at 95°C, followed by 32 PCR cycles of 50 seconds at 95, 40 seconds at 68, and 30 seconds at 72°C, and a final 4 minute extension at 72°C. In order to analyze the PCR product, 5 µl of the reaction product was electrophoresed on a 1.5% agarose gel. The presence of a 235-bp band indicated a successful amplification of the Rifr region. Precautions to avoid cross-contamination and false-positive results were taken in every assay.<sup>20</sup>

**Single-strand conformation polymorphism analysis.** The SSCP analysis was performed by mixing 5 µl of the PCR product 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 5 minutes at 98°C, cooled on ice for 10 minutes, and loaded onto a sequencing format of 4% acrylamide gel and electrophoresis was performed at room temperature overnight at constant power (100 volts for a gel of 21 by 22 by 0.1 cm). The gels were stained with silver nitrate. The rifampin-sensitive isolate *M. tuberculosis* H37rv (ATCC 27294) and 3 rifampin-resistant isolates of *M. tuberculosis* with different mutations located in the Rifr region were used as controls.

Ethical approval for the study was obtained from the Regional Ethical Committee.

**Results.** In 24 patients, diagnosis was confirmed by direct microscopic examination or culture. A positive



**Figure 1** - Showing polymerase chain reaction-single strand conformation polymorphism patterns obtained by processing 7 clinical isolates, a rifampin-resistant *Mycobacterium tuberculosis* (R) and a rifampin-sensitive *Mycobacterium tuberculosis* reference strain (H37Rv).

result was obtained for 20 CSF samples from these patients with tuberculosis meningitis by the IS6110 PCR. Testing of susceptibility to rifampin was carried out for 20 of these strains by the use of a conventional method, which revealed a sensitive phenotype. Amplified products suitable for subsequent SSCP analysis were obtained from 16 CSF samples by the *rpoB* PCR. The 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 all 16 samples (Table 1). Among these patients, no different SSCP patterns were identified (Figure 1). The SSCP analysis did not reveal any *rpoB* gene mutations in the genomes of the mycobacterial isolates from the CSF of these patients. Table 1 summarizes the results obtained by processing 47 CSF samples suffering from tuberculosis meningitis by using the 2 PCR protocols, SSCP analysis of the amplified products of *rpoB*, and testing of the susceptibilities of the clinical isolates to rifampin. In this study, among 47 patients the CSF samples collected from 23 patients were negative upon culture, PCR, and microscopic examination. Figure 1 shows the SSCP patterns obtained by processing CSF samples and the rifampin-sensitive strain *M. tuberculosis* H37Rv (ATCC 27294) as control, also a rifampin-

**Table 1** - Analysis of CSF samples, results obtained by 2 PCR protocols, patterns by PCR-SSCP, and susceptibility of clinical isolates to rifampin.

No. of patients	Mean Age (years)	Gender (Male/Female)	No. of samples positive by		Pattern by PCR-SSCP analysis	Rifampin phenotype
			Microscopic exam or culture	IS6110 PCR <i>rpoB</i> PCR		
47	37.4 (±22.9)	22/25	24	20 16	All wild type	All sensitive

CSF – cerebrospinal fluid, PCR - Polymerase chain reaction, SSCP – single-strand conformation polymorphism, SSCP patterns were classified as wild type in the case of DNA strands with electrophoretic mobilities identical to that of a reference strain (*Mycobacterium tuberculosis* H37rv [ATCC 27294]).

resistant *M. tuberculosis* isolate with mutation located in the Rifr region, which was used as controls were observed in this figure.

**Discussion.** In this study, among 24 positive samples, 8 specimens were not detected with *rpoB* PCR method and 4 specimens were not detected with IS6110 PCR method (sensitivity 66.6% and 83.3%), this is probably due to poor extraction of DNA directly from clinical samples. As 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. It is for this reason that, in this study like many other studies, the amplification of Rifr at the genomic level was only possible with less samples compared to IS6110 PCR.<sup>12,21</sup> Whelen et al<sup>16</sup> 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. Regarding this point, for obtaining the best result, in addition to usage of better protocols, a combination of microscopic examination, culture, and PCR would be recommended.

The use of molecular biology-based techniques in the search for rifampin resistance 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 approach,<sup>11-17</sup> although some unresolved methodological problems are still encountered when DNA is extracted directly from clinical samples.<sup>16</sup>

The present paper reports the results of the SSCP analyses of *rpoB* gene amplification products obtained by processing CSF samples from subjects with tuberculosis meningitis. Amplification products suitable for the subsequent detection of mutations were obtained from the CSF samples drawn from 16 (66.6%) of the 24 subjects whose tuberculosis meningitis was confirmed by means of microbiological investigations. Therefore, SSCP analysis was possible for 66.6% of the patients.

In our experience, the detection of rifampin susceptibility by genotypic study of the mycobacterial DNA present in 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 obtained from these 16 patients with tuberculosis meningitis showed a rifampin phenotype that was in line with the results of the SSCP analysis. For 8 patients, it was not possible to compare

the rifampin phenotype with the SSCP data because no *rpoB* gene amplification products were available for these patients.

Kim et al<sup>22</sup> used nested PCR-linked SSCP and DNA sequencing for detection of rifampin-resistant *M. tuberculosis* directly in sputa, and the obtained results were concordant with those of conventional drug susceptibility testing and DNA sequencing performed with culture isolates. Scarpellini et al<sup>23</sup> used a similar method for direct detection of rifampin resistance in the CSF of patients with tuberculosis of the central nervous system, and PCR-SSCP analysis of CSF seems to be an efficacious method of predicting Rifr and would reduce the time required for susceptibility testing from approximately 4-8 weeks to a few days.<sup>23</sup> These studies both confirmed the findings of our study.

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. A complete cure was obtained for 26 patients, whereas in 10 patients no response was observed, or they died within 10 days of the onset of the disease, and 11 patients showed incomplete improvement (data not shown).

In conclusion, SSCP analysis of *rpoB* gene amplification products obtained from mycobacterial DNA taken from the CSF of subjects with tuberculosis meningitis show complete susceptibility to rifampin. Its use can radically reduce the time needed to provide clinicians with data useful in aiding the selection of appropriate drugs for the efficacious treatment of tuberculosis.

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#### Related topics

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