Use of Allele-Specific Amplification and Analysis of Conformation Polymorphism for Detecting Rifampicin Resistance of Clinical Strains of *Mycobacterium Tuberculosis*

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Analysis of 90 clinical strains of *M. tuberculosis* demonstrated the possibility of using allele-specific amplification for detecting mutations determining rifampicin resistance. Successive use of two allele-specific primers helped us to detect 85% resistant strains. Other strains were studied using conformation polymorphism analysis of single-stranded DNA fragments. Consecutive use of two assays correctly identified 98% resistant strains.

Key Words: *mycobacterial genome; point mutations; rifampicin resistance of M. tuberculosis*

Effective treatment of tuberculosis depends on early detection of drug resistance. Routine microbiological tests for detecting drug resistance are the most accurate, but they take from 3 weeks to several months, which makes them inappropriate. An alternative approach to this problem is the use of molecular genetic methods detecting drug resistance within several days. This approach has been most effectively realized in the diagnosis of *Mycobacterium tuberculosis* resistance to rifampicin, the main drug used in antituberculosis therapy [8]. Such diagnosis is based on detection of point mutations located in the *rpoB* gene encoding β-subunit of bacterial RNA-polymerase responsible for rifampicin resistance [8]. These mutations are usually detected by direct DNA sequencing of amplified *rpoB* gene fragments [7,8], reverse hybridization with oligonucleotide probes [2], and conformation polymorphism analysis of single-stranded DNA fragments (SSCP) [4,5]. However these methods are expensive and labor-consuming, which impedes their use in routine diagnosis.

We investigated the possibility of detecting mutations in the *rpoB* gene by a simple method of allele-specific amplification (ASA) [3]. The proposed combined strategy for detecting rifampicin-resistant strains includes ASA which detects up to 80% resistant strains and subsequent analysis of the rest strains by SSCP.

MATERIALS AND METHODS

A total of 90 clinical strains of *M. tuberculosis* were examined, 46 of these were resistant and 44 were sensitive to rifampicin. The strains were isolated from patients with pulmonary tuberculosis. The sensitivity of mycobacteria to the main antituberculosis drugs (rifampicin, isoniazide, streptomycin, kanamycin, ethambutol, and pirasinamide) was evaluated by the method of absolute concentrations.

The cultures were inactivated by heating at 95°C for 10 min, after which DNA was isolated as described previously [1].

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A fragment of *rpoB* gene (157 b. p.) containing the rifampicin resistance region was amplified using *rpo*8 (5"TGCACGTCGCGGACCTCCA3') and *rpo*9 (5TCGCCGCGATCAAGGAGT3') primers. The reaction was performed in 25 μl reaction mixture containing 66 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 250 μM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 U Taq-polymerase (Promega). Thirty amplification cycles were carried out: denaturation at 94°C for 1 min (2 min in the first cycle), annealing at 60°C for 1 min, and synthesis at 72°C for 1 min (10 min in the last cycle).

ASA was carried out with *sp*1 (5"ACCCACAAG CGCCGACTCTC3') or *sp*2 (5"CGACAGCGGGTT GTTCTCGA3') primers on an amplicon template synthesized previously with *rpo*8 and *rpo*9 primers. Mutations in codons 526 and 531 of the *rpoB* gene were detected during amplification with primers *sp*1 and *rpo*8, and mutations in codon 516 during amplification with primers *sp*2 and *rpo*9. The amplification procedure and reaction mixture were the same as described above, the number of cycles was decreased to 12.

A 157 b. p. fragment obtained using *rpo*8 and *rpo*9 was used in SSCP. The amplification product was diluted 6-fold with formamide, heated at 55°C for 4 min, and transferred into rows of 18.5% PAAG of the following composition: Tris-borate buffer (Tris 89 mM, boric acid 89 mM, pH 8.0), acrylamide/bisacrylamide (30:1), 0.002 M EDTA, and 8.3% glycerol.

Electrophoretic separation of denatured DNA was performed in a Mini-Protean II device (Biorad) plunged in a water tank cooled to 10-18°C. Electrophoresis was carried out for 12-15 h at 150 V. The gel was stained with silver nitrate [6].

RESULTS

Rifampicin resistance mutations in *M. tuberculosis* strains were most frequent in codons 531, 526, and 516 of *rpoB* gene [8,9]. The strains carrying these mutations constitute about 80% of the total number of resistant strains. The procedure for detecting resistant strains proposed by us is adapted primarily for detecting strains with these mutations.

The procedure includes 4 stages.

The first stage consisted of amplification of 157 b. p. fragment of *rpoB* gene containing the rifampicin resistance domain (Fig. 1, stage I). Amplification product was diluted to a concentration of about 2 ng/μl and used as a template for ASA with primers *sp*1 and *rpo*8 (Fig. 1, stage II).

Primer *sp*1 is identical to the sequence of rifampicin sensitive strains in the region of codons 526 and 531 of *rpoB* gene, except the third 3' terminal nucleotide. The presence of mutations in codons 526 or 531 renders amplification imposible (M—), while amplification of the DNA fragment from sensitive strains yields a 80 b. p. product (M+).

At the next stage the samples in which no mutations were detected in codons 531 or 526 were amplified with primers *sp*2 and *rpo*9 (Fig. 1, stage III). This primer is complementary to a frequently occurring mutant carrying A-T substitution in the second position of codon 516. The presence of this mutation in the examined sample is associated with amplification of a 71 b. p. product (M+). If the mutation is absent, no amplification occurs (M—). Finally, the samples in which no mutations were found in codons 516, 526, and 531 were analyzed by SSCP (Fig. 1, stage IV).

Thus, the proposed protocol for detecting mutations allows to detect the majority of resistant strains during the first stages, thus minimizing the number of strains to be evaluated by a more sophisticated SSCP method.

For evaluating the efficiency of the proposed combination of methods we analyzed 44 sensitive and 46 resistant clinical strains of *M. tuberculosis*. The data of ASA and SSCP were compared with the results of direct sequencing of *rpoB* gene in the same samples.

ASA and direct sequencing of 46 strains phenotypicallly resistant to rifampicin detected 39 (85%) strains with mutations in *rpoB* codons 531, 526, and 516; of them, 33 strains were detected using *sp*1 primer and 6 with *sp*2 primer. Six resistant strains were analyzed by SSCP. Only one of 46 phenotypically resistant strains was not correctly identified by ASA and SSCP. Sequencing of the respective fragment of *rpoB* from the same sample also showed no mutations between codons 511 and 533. Presumably, this strain possesses an alternative mechanism of rifampicin resistance, which does not depend on *rpoB* gene.

No mutations were found by ASA in 44 strains sensitive to rifampicin.

Thus, analysis of different clinical strains of *M. tuberculosis* by ASA with two primers detects about 85% and ASA in combination with SSCP detects 98% resistant strains.

Effects of primers *sp*1 and *sp*2 virtually do not depend on the concentrations of initial DNA (1-4 mg/μl) and number of amplification cycles (±2 cycles). It is noteworthy that primer *sp*1 is virtually universal. Sequencing of resistant strains showed that this primer correctly identified at least 5 types of mutants, of them 4 mutations were located in codon 526.

The most common kits for detection of mutations in *rpoB* gene of *M. tuberculosis* INNO-LiPa Rif.TB (Innogenetics) and Mis-Match Detect II (Ambion) are expensive and require special training. ASA includes only two simple methods: amplification and agarose gel electrophoresis. Samples not identified by ASA are

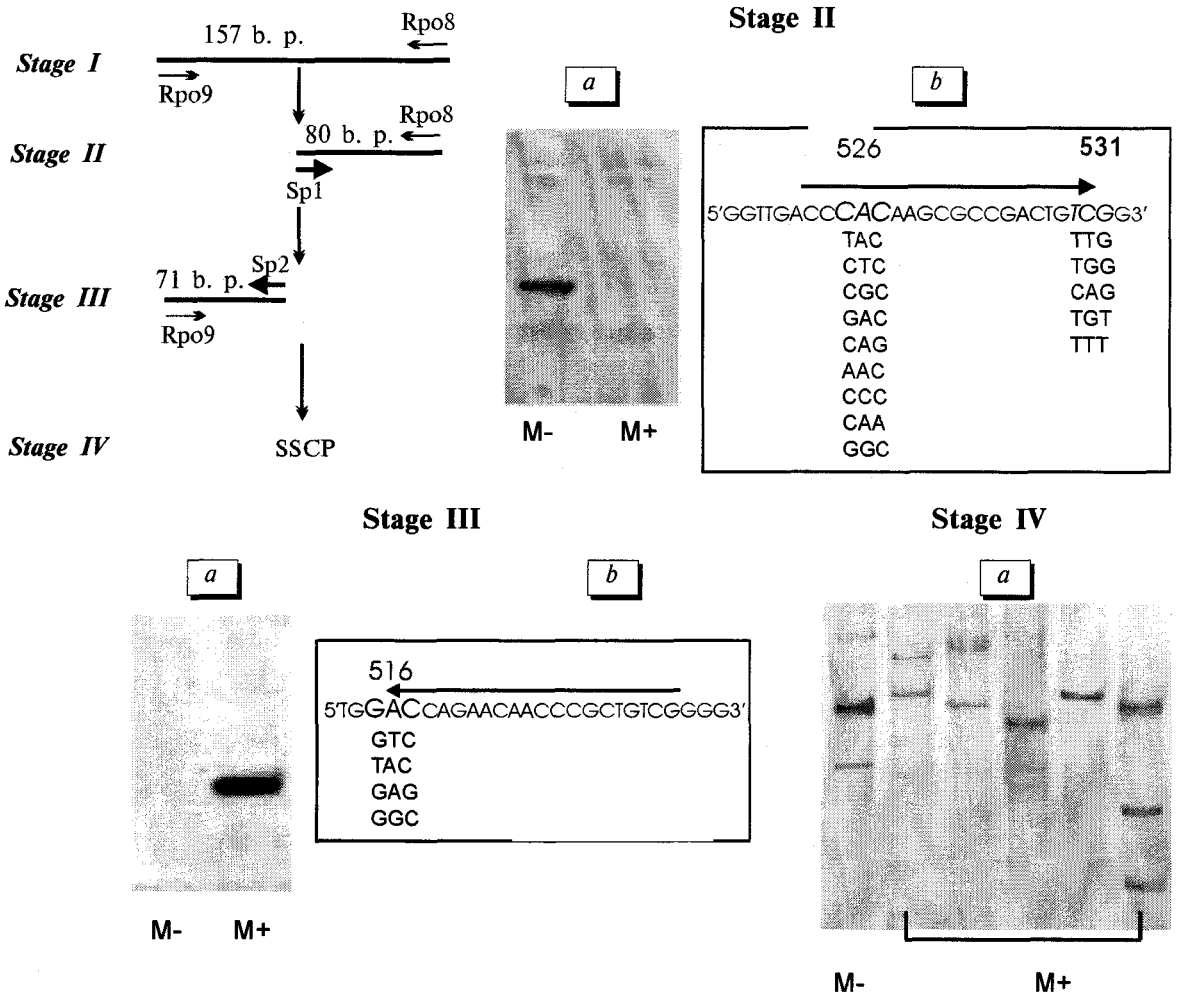

Fig. 1. Detection of mutations in *rpoB* gene of *Mycobacterium tuberculosis* by allele-specific amplification with primers *sp*1 (stage II) and *sp*2 (stage III) and conformation polymorphism analysis of single-stranded DNA fragments (stage IV). Left: scheme of experiment. *a*) Fragments of electrophoregram. M+ samples with mutation, M— samples without mutations; *b*) sequences of *rpoB* gene representing annealing sites of *sp*1 and *sp*2 primers. Codons 516, 526, and 531 are shown with large letters. Known mutations in these codons responsible for rifampicin resistance are shown below.

analyzed by SSCP in a portable economic device for vertical electrophoresis.

Hence, detection of rifampicin-resistant strains of *M. tuberculosis* by ASA is very effective, but limited by the stage of preparation of primary *M. tuberculosis* culture. Elimination or reduction of culturing to several days will increase the practical value of the proposed method.

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