Ratio of Loading Dye to PCR Product Affects Sensitivity of Single-Stranded Conformational Polymorphism Analysis

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Rifampin resistance in *Mycobacterium tuberculosis* involves missense mutations, small insertions, or deletions (Table 1 and References 1 and 2) in a well-characterized region of the *rpoB* gene encoding the β -subunit of the RNA polymerase (2). These mutations have been found in more than 97% of rifampin-resistant *M. tuberculosis* strains (3) and are located in the 81-bp-long region of the *rpoB* gene. Single-stranded conformational polymorphism (SSCP) is a method that can detect these kind of mutations and has been evaluated as a potential tool for the genotypic detection of rifampin-resistant *M. tuberculosis* by several authors (1,4-6).

Different parameters can affect the mobility shift in SSCP. These are temperature, ionic strength, the composition of the gel, and additives (7). The type of base change, base composition, and the size of the DNA fragment (8) are other important parameters that play a role in the sensitivity of SSCP. Here we report the enhanced sensitivity of SSCP by decreasing the loading dye to PCR product ratio.

A 305-bp fragment of the *rpoB* gene (2) of rifampin-resistant *M. tuberculosis* strains, which spans the region of 81 bp, was amplified and subjected to SSCP analysis. H₃₇Rv strain (reference no. 27294; ATCC, Manassas, VA, USA), which is sensitive to rifampin, was used as control. The following SSCP conditions were used: 20°C, with cooling provided by a water jacket; 200 V; 0.5× TBE buffer (45 mM Tris, pH 8.3, 45 mM boric acid, 1 mM EDTA at 25°C) and 10% acrylamide gel (cross-linking ratio = 3%; total acrylamide percentage = 30%). A MightySmall SE 260 (Hoefler Scientific Instruments, San Francisco, CA, USA) vertical slab gel unit was used (100 × 105 × 0.75 mm gels). The 7-μL PCR product was mixed with 1, 3.5, or 7 μL SSCP load-

Table 1. Sequence Analysis of the *rpoB* Gene of Rifampin-Resistant *M. tuberculosis* Strains

Strains		
Missense mutation	59 (89.4%)	98 (89.1%)
Ser531Leu mutation*	31 (47.0%)	46 (41.8%)
His526Tyr mutation*	8 (12.1%)	20 (18.2%)
Two point mutations	4 (6.0%)	2 (1.8%)
Insertion (3 bp)	—	1 (0.9%)
Deletion (3 bp)	1 (1.5%)	1 (0.9%)
No mutation	2 (3.0%)	8 (7.3%)
Total	66	110

These data are from References 1 and 2, together with the two most frequent mutations (indicated by *).

ing dye [(95% formamide; Serva, Heidelberg, Germany), 20 mM EDTA (Merck, Darmstadt, Germany), 0.25% bromphenol blue, and 0.25% xylene cyanol (both from Serva)], boiled for 5 min, placed on ice, loaded onto the gel, and electrophoresed for 120 min. The gels were stained by silver staining. Figure 1 shows that, by varying the loading dye to PCR product ratio from 1:7 to 1:1, we produced different SSCP patterns in the H₃₇Rv strain on given electrophoretic conditions. Multiple bands that were seen at a 1:7 ratio were reduced to three bands at a 1:1 ratio, the fastest being double-stranded, renatured DNA. To compare the sensitivity of SSCP on these two conditions, a sample of 39 rifampin-resistant *M. tuberculosis* strains collected in Serbia was used. The electrophoresis time was 120 min for the samples mixed with 1 μL loading dye and 240 min for those mixed with 7 μL loading dye. SSCP with a 1:7 ratio, compared to a 1:1 ratio, detected mutations in 10.3% more of the strains. Figure 2 shows examples of SSCP profiles for strains for which the change in electrophoretic mobility was detected only with the 1:7 loading dye to PCR product ratio. Multiple bands contributed to the informativeness of SSCP (Figure 2B). Especially, the change in mobility was frequently seen for the band nearest the renatured DNA, the band that did not appear when 1:1 ratio was used. The nature of this band is unknown. Considering the position of the band in the gel, we can assume that a decreased amount of denaturant formamide allowed some kind of reannealing to occur. Because the

PCR product was not purified before SSCP analysis, excess primers may reanneal with ssDNA prior to entry into the gel. If this is the case, then samples that do not have this band (Figure 2B, lanes 1 and 3) might have mutations that are unfavorable for the reannealing of the primer, in spite of the decreased amount of formamide. In those strains that show a shift of this band, compared to H₃₇Rv (Figure 2B, lanes 5 and 6), the mutation may act in such a way that reannealing still occurs but is accompanied with a change in conformation and electrophoretic mobility of that conformer. We sequenced a 81-bp-long region of the *rpoB* gene of one strain that showed small changes in the SSCP profile on a 1:1 dilution and a shift in the band nearest the dsDNA on a 7:1 dilu-

Figure 1. Change in SSCP profiles with increasing dilutions of PCR product. The 7-μL PCR product of *M. tuberculosis* H₃₇Rv was mixed with either 1, 3.5, or 7 μL loading dye.

Figure 2. Comparison of SSCP analysis for two electrophoretic conditions. (A) A 1:1 loading dye to PCR product ratio. (B) A 1:7 loading dye to PCR product ratio. H₃₇Rv was used as control (lane H). Rifampin-resistant *M. tuberculosis* strains with mobility shift detected only with the 1:7 ratio (lanes 1, 5, and 6). The low yield of PCR product, which is clearly visible on SSCP with the 1:1 ratio, is barely visible on the 1:7 ratio (wild-type SSCP profile on both conditions, lane 2; shift detected on both conditions, lane 3). Blurred band on both dilutions of the PCR product (lane 4).

tion of the PCR product. A C→T transition was found at position 526. The same transition, but at position 531, was found in another strain that we sequenced that had wild-type SSCP profiles on both dilutions of the PCR product. This mutation (Table 1) was reported to make only subtle changes in migration on the mutation detection enhancement gel (4) and, obviously, leads to very small changes in conformation of ssDNA, which did not cause any changes in electrophoretic mobility on our SSCP conditions. Since (in-frame) insertions and deletions are very rare in this fragment of the *rpoB* gene of rifampin-resistant *M. tuberculosis* strains (Table 1), it is very possible that most of the other strains, if not all of them, including those 10.3%, are with missense mutations. It should also be mentioned that, for two patients, SSCP analysis was performed for two isolates. In both cases, the SSCP analysis of the second isolate showed the same patterns as the first isolate that had been obtained several months earlier (see Figure 2, lane 6).

Hongyo et al. (9) studied the impact

of loading solutions on SSCP. They proposed the 1:4 dilution of the PCR product and found that dilutions of less than 3:6 resulted in significant amounts of dsDNA and blurred bands in the SSCP gel. We found that the amount of dsDNA increased with increasing dilutions of the PCR product (Figure 1). Blurred bands were detected in two strains (5.1%, possibly with the same mutation), on both dilutions of the PCR product. The clear change in mobility was seen only when a 1:1 dilution was used (Figure 2). In addition, while multiple bands were barely visible when the amount of PCR product was smaller, the two bands on the 1:1 ratio were clearly visible (Figure 2).

In this collection of rifampin-resistant *M. tuberculosis* strains, the 1:7 loading dye to PCR product ratio enhanced SSCP sensitivity, compared to the 1:1 ratio, by 10.3%. Therefore, decreasing the dilution of the PCR product should also be considered as a parameter that can increase the sensitivity of SSCP on certain electrophoretic conditions and enable the detection of some of the shifts that lacked when an equal ratio was used.

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