Single strand conformation polymorphism profiles with biotinylated PCR products to detect mutations in *rpoB* gene of *Mycobacterium tuberculosis*

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A fragment of the *rpoB* gene, including the region shown to be involved in rifampicin resistance, was amplified from 15 rifampicin-resistant and 6 rifampicin-sensitive clinical isolates of *Mycobacterium tuberculosis* by the polymerase chain reaction (PCR). One of the primers, employed in PCR, was biotinylated. The biotinylated strand of the PCR product was separated from the unbiotinylated strand using streptavidin magnetic beads. Both the strands were subjected to single strand conformation polymorphism in polyacrylamide gel electrophoresis. The DNA bands were silver stained to study their migration pattern. A shift in the migration of either of the strands of the test strain compared to the strands from a control rifampicin-sensitive strain was considered as indicative of resistance. This strategy was found to ease the visualization of shift in the migration of the strands in 17 of 21 samples and thereby detection of mutations.

SINGLE strand conformation polymorphism (SSCP) is one of the usual procedures to detect mutations[1,2]. It is based on the fact that separated strands of DNA adopt a folded

conformation as a result of self-complementarity and intramolecular interactions. A single nucleotide mutation usually leads to an altered conformation that can be identified as a change in DNA strand mobility by nondenaturing gel electrophoresis. Therefore, in SSCP, a good separation of DNA strands is essential to achieve sharp migration of bands. Often, DNA strands reanneal rapidly to form double strand DNA with little or no single DNA strand for SSCP analysis[3]. Biotinylation of one of the primers employed in the PCR and subsequent separation of the biotinylated strand from the unbiotinylated strand using streptavidin magnetic beads prevented the reannealing of strands and was found to ease the detection of shift in the migration of bands[4]. However, in the above study, a nested PCR was employed to amplify the region of the *rpoB* gene in which mutations have been shown to determine the rifampicin resistance. In the first PCR, a 293-bp region of the *rpoB* region of *Mycobacterium tuberculosis* was amplified. In the second PCR, 103-bp region of the first PCR product was amplified using a biotinylated forward primer. It is evident that the nested PCR requires more time and is more expensive. So, it is always desirable to have a simple and less expensive procedure which is practicable. Therefore, the aim of this study was to generate a biotinylated PCR product in a single PCR using a biotinylated forward primer and an unbiotinylated reverse primer for easy detection of mutations.

The clinical isolates were obtained from the pulmonary tuberculosis patients attending Tuberculosis Research Centre, Chennai. Fifteen rifampicin-resistant isolates and six rifampicin-sensitive isolates were selected and coded. One rifampicin-sensitive clinical isolate of *M. tuberculosis* was used for reference.

A portion of *rpoB* gene which is associated with rifampicin resistance in *M. tuberculosis,* was amplified by PCR. The PCR mixture (20 µl) contained 50 mM potassium chloride, 10 mM Tris-hydrochloric acid pH 8.3, 1.5 mM magnesium chloride, 5% dimethysulphoxide, 20 µM each of dATP, dGTP, dCTP, dTTP, 10 pM each of a biotinylated forward- *rpoB* FI Bio (5' GT TCT TCG GCA CCA GCC AG 3') and an unbiotinylated reverse-*rpoB* RO (5' TTT CGA TGA ACC CGA ACG GGT TGA C 3') primer (the primers were synthesized at R & D Systems Europe Ltd, UK) and 1 unit of Taq polymerase (Bio line). The DNA was extracted from the cultures by the procedures described by Baess[5], and was used as template. The PCR was carried out using 0.5 ml microcentrifuge tubes in a Hybaid Omni Geni thermo reactor. The reaction mixture was denatured at 93°C for 2 min followed by 35 cycles each of denaturation at 93°C for 30 sec annealing at 58°C for 30 sec and extension at 72°C for 30 sec. The reaction was terminated after a final extension at 72°C for 10 min. The products were checked on 2% agarose gel electrophoresis using the standard procedures.

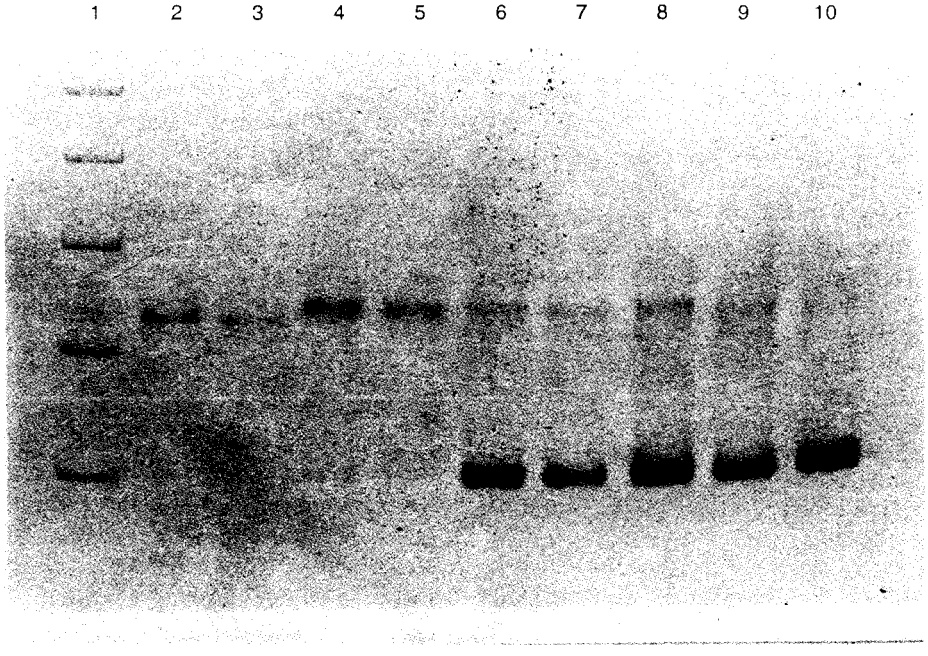

Figure 1. PCR-SSCP profiles of UBS and BS from a rifampicin-sensitive and rifampicin-resistant isolate of *M. tuberculosis.* Lane 1, DNA marker; lane 2, Biotinyluted rif. resistant; lane 3, Biotinylated rif. sensitive; lane 4, Unbiotinylated rif. resistant; lane 5, Unbiotinylated rif. sensitive; lane 6, Alkali denatured rif. resistant; lane 7, Alkali denatured rif. sensitive; lane 8, Heat denatured rif. resistant; lane 9, Heat denatured rif. sensitive; lane 10, Undenatured double-stranded DNA (control).

The separation of biotinylated strand by streptavidin magnetic beads was carried out according to instructions from the manufacturer, Dynal, UK. In brief, 40 µl of the beads, suspended in 2x binding and washing buffer (BW buffer) pH 7.5 (10 mM Tris, 1 mM ethylene-diaminetetraacetic acid (EDTA) and 2 M sodium chloride) were mixed with equal volume of PCR product and incubated at room temperature for 30 min. After washing the beads with 1 x BW buffer, the captured DNA was denatured by adding 8 µl of 0.1 M sodium hydroxide (NaOH) and incubated for 10 min at room temperature. The alkali containing the unbiotinylated strand (UBS) was aspirated, made up to a volume of 50 µl in Tris-EDTA (TE) buffer (pH 8.0) and then precipitated by a standard ethanol-sodium acetate method. This strand was resuspended in 5 µl of TE buffer. The beads with the captured biotinylated strand (BS) were washed once with 50 µl of 0.1 M NaOH and washed 3 times with 1 x BW buffer and finally suspended in 5 µl of TE buffer. The separated strands were heated at 95°C for 5 min with an equal volume of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% formamide) and then snap-cooled before immediately loading onto the gel.

A 10% gel was prepared by mixing 10.5 ml of 40% acrylamide-bisacrylamide (49 : 1; Sigma), 27.4 ml of deionized water, 2.1 ml of 10x Tris borate EDTA buffer (TBE buffer) pH 7.6, 2 ml of glycerol, 21 µl of tetramethylethlenediamine and 210µl of 10% ammonium persulphate[6]. The dimension of the gel was 180 x 160 x 1.6 mm. The electrophoresis was carried out in Hoefer 600 model using 0.5 x TBE buffer at 90 V for 15 to 18 h at room temperature. The separated DNA strands were visualized by silver staining.

Table 1. Observations on the mobility of DNA strands and the results of the classification of rifampicin sensitivity in 21 samples

Sample no.	Mobility of		Classification by	
	UBS*	BS	PCR-SSCP	Bacteriology
	Slow	Slow	R	R
3.	Slow	Equal	R	R
4.	Equal	Equal	S	R
6.	Slow	Equal	R	S
7.	Fast	Fast	R	R
8.	Equal	Fast	R	R
9.	Slow	Slow	R	R
11.	Equal	Equal	S	S
12.	Equal	Equal	S	S
13.	Equal	Equal	S	S
19.	Slow	Equal	R	R
20.	Slow	Equal	R	S
21.	Slow	Equal	R	S
22.	Slow	Equal	R	R
23.	Undetermined	Fast	R	R
32.	Fast	Fast	R	R
33.	Slow	Slow	R	R
34.	Slow	Slow	R	R
36.	Slow	Slow	R	R
38.	Undetermined	Fast	R	R
44.	Undetermined	Fast	R	R

*UBS: Unbiotinylated strand; BS: Biotinylated strand; R: Resistant; S: Sensitive.

The gel was treated with ethanol (10%), nitric acid (1%) and silver nitrate (0.2%)-formaldehyde (0.1%) solutions, respectively for 5, 3 and 20 min. Later the gel was washed three times with double distilled water before it was treated with sodium carbonate (3%)-formaldehyde (0.025%) solution to stain the DNA. After staining[7], the gel was preserved in glacialacetic acid (10%) and photographed.

The slow or fast migration of either of the strands, compared to the corresponding strands from the rifampicin-sensitive reference control, was considered as indicative of rifampicin resistance.

The PCR-SSCP profiles of UBS and BS from a rifampicin-sensitive and a rifampicin-resistant isolate of *M. tuberculosis* are shown in Figure 1. Both the UBS and BS of the rifampicin-resistant strain migrated slower than the corresponding strands of the reference rifampicin-sensitive strain. It is also seen that the alkali and heat denaturation of PCR products resulted in reannealing of strands with little DNA for separation. The observations of the migration patterns of UBS and BS in 21 samples are given in Table 1. In 3 samples (nos 23, 38, 44), the migration of UBS was not determined either due to poor staining of little or loss of DNA. It can be noted that 13 of UBS and 11 of BS showed difference in their migration pattern compared to the corresponding control strands. Eleven of 18 UBS migrated slowly compared to 5 of 21 BS. Only 2 of 18 UBS migrated fast while 6 of 21 BS did so. In 7 samples the difference in migration pattern was exhibited by both UBS and BS and in another 7 samples it was by either of the strands. In this study, 14 of 15 rifampicin-resistant strains and 3 of 6 rifampicin-sensitive strains were correctly identified by the PCR-SSCP.

Factors that affect SSCP analysis are discussed by Yap and McGee[3]. One of the factors, that is the re-annealing of the strands, especially when the product size is less than 100 bp, reduces the efficiency of SSCP analysis[3,4]. The reannealing of strands can take place while the samples are being loaded and during the initial period of electrophoresis before the DNA has entered the ge1[3]. Yap and McGee[3] had mentioned that the alkali denaturation resulted in better separation of single strand DNA because the alkali prevents the reannealing of strands. They also stated that heating at 42°C for 10 min before loading onto the gel reduced reannealing on some occasions. Both these were tried in our samples but we failed to get better separation. In order to overcome this, a novel approach was attempted. In this, a biotinylated primer was used in PCR. Then, the biotinylated and the unbiotinylated strands were separated and sub-

jected to SSCP by PAGE. The recognition of shift in the migration of strands was thus made easy. Another factor, that is the multiple conformations of strands would also limit the efficiency of PCR-SSCP analysis. The SSCP analysis of the PCR products of all our samples produced 4 bands in a different gel format and protocol (data not shown). This could be attributed to the possible two conformations of each of the strands while the other possibilities cannot be ruled out. Similarly, Telenti *et al.* [8] observed a three-band pattern in his samples. In the present study, the occurrence of the multiple conformations of strands was not observed although it remains to be explained.

A large-scale study to determine the association of the migration pattern of single stranded DNA with the specific nucleotide change in the *rpoB* gene might be useful for the presumptive identification of specific mutants in the clinical isolates. It is interesting to note that Telenti *et al.* [9] observed a specific migration pattern for each of the nucleotide substitution.

In this study, 3 (nos 6, 20, 21) of 6 rifampicin-sensitive strains were misclassified by the PCR-SSCP. It should be realized that SSCP does not differentiate rifampicin-sensitive strains with functionally silent sequence changes. Therefore, DNA sequencing of the PCR products only could confirm the mutations occurring in these 3 specimens. Also, 1 (no. 4) of 15 rifampicin-resistant strains was misclassified by PCR-SSCP in the present study. On scrutiny, this isolate was obtained from a patient whose alternative isolates were sensitive to rifampicin. It should be pointed out that using different protocols such as conventional PCR-SSCP[8] and automated sequencing[10], the variations in the classification were reported. In the former, 2 of 66 and in the latter 3 of 121 rifampicin-resistant strains were misclassified.

The present PCR-SSCP format takes less time and is less expensive as it involves only one PCR. The results suggest that this procedure can be adopted for the detection of mutations in the *rpoB* region of *M. tuberculosis*. However, a separate study using a large number of sensitive and resistant strains needs to be carried out to assess the validity of the method. Also, attempts should be made for the early detection of rifampicin-resistant *M. tuberculosis* in sputum samples of pulmonary tuberculosis patients as it is a surrogate marker of multidrug-resistant tuberculosis.

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