USE OF STREPTAVIDIN MAGNETIC BEADS IN SINGLE STRAND CONFORMATION POLYMORPHISM PROFILES TO DETECT MUTATIONS IN rpoB GENE OF M.TUBERCULOSIS.

Selvakumar N*, Ding BC, Ruth McNerney,*****
Stuart M Wilson* and Narayanan PR.***

***Tuberculosis Research Centre, Chennai 600 031, India.**
****Beijing Research Institute for Tuberculosis Control, Beijing, 100 035 P.R. China.**
*****London School of Hygiene and Tropical Medicine, London, WC 1E 7HT.**

Introduction

Single strand conformation polymorphism (SSCP) is one of the promising techniques to identify mutations in short pieces of DNA (Orita *et al.* 1989). In this technique, DNA of interest is often amplified by the polymerase chain reaction (PCR) and then denatured by heat or alkali treatment before electrophoresis on a non denaturing polyacrylamide gel. Differences in mobility of either of the single strands compared to the control DNA indicate mutations which affect the secondary structure and alter the mobility of the DNA. We applied PCR-SSCP for the detection of mutations in the rifampicin resistance determining region (RRDR) of the *rpoB* gene of *M. tuberculosis* (Telenti *et al.* 1993a; 1993b). A nested PCR was used to amplify the RRDR. In the first PCR, 293-bp product was amplified and in the second PCR a 103-bp of the first PCR product was amplified. However, in our experience using denaturation by alkali or heating, the denatured PCR product most often reannealed to form a large proportion of double stranded DNA during the electrophoresis (Selvakumar *et al.* 1997a). After visualisation by staining with ethidium bromide or silver staining, most of the DNA was in the double stranded form, with very little or no single stranded DNA. The single strands that could be observed often ran close together, making analysis of any difference in mobility difficult. Therefore an attempt was made to generate biotinylated PCR product using a biotinylated forward primer and later the biotinylated strand was separated using sterptavidin magnetic beads. The separated strands eliminated the problem of strand reannealing during SSCP and were silver stained to detect the shift in the mobility. Since the nested PCR requires more time and is more expensive. a biotinylated PCR product was generated in a single PCR using a biotinylated forward primer and an unbiotinylated reverse primer. This simplified protocol was applied to clinical isolates in an attempt to detect rifampicin resistance (Selvakumar *et al.* 1997).

Materials and Method

Clinical isolates:

The clinical isolates were obtained from the pulmonary tuberculosis patients attending Tuberculosis Research Centre, Chennai. Fifteen rifampicin resistant isolates and 6 rifampicin sensitive isolates were selected and coded. One rifampicin sensitive clinical isolate of *M. tuberculosis* was used as reference.

Extraction of DNA:

The DNA was extracted from the cultures by the procedures described by Baess (1974) and was used as template. The PCR was carried out using 0.5 ml microcentrifugc tubes in a Hybaid Omni Geni thermo reactor.

Nested PCR:

First PCR: Two hundred and ninety three base pairs comprising of RRDR of the rpoB gene was amplified by a first PCR. The PCR mixture (20 ul) contained 50 mM Potassium chloride (KCl), 10 mM Tris-Hydrochloride (Tris - HCl; pH 8.31, 1.5 mM Magnesium chloride (MgCl), 5%, Dimethyl sulfoxide (DMSO), 200 ul (each) of dATP. dGTP, dCTP and dTTP, 10 pM each of a forward- rpo B FO (5' **CGT TGA TCA ACA TCC GGC CGG TGG** 3') primer and a reverse- rpo BRO (5' **TTT CGA TGA ACC CGA ACG GGT TGA C** 3') primer and 1 unit of Taq polymerase (BioTaq). The reaction mixture was denatured at 93°C for 2 minutes followed by 35 cycles denaturation (93° C) , annealing (58°C) and extension (72°C) C for 1 minute each. The reaction was terminated after a final extension at 72°C for 10 minutes.

Second PCR:

One hundred and three base pairs of the first PCR product was amplified in the second PCR. The reaction mixture contained the same ingredients as above except for the biotinylated forward- rpo B FIBio (5' **GT TCT TCG GCA CCA GCC AG** 3') and unbiotinylated reverse- rpo B SRI (5' **CAG ACC GCC GGG C CC** 3') primers. The reaction mixture was denatured at 93°C for 2 minutes followed by 20 cycles of denaturation (93°C), annealing (52°C) and extension (72°C) for 30 seconds each. The reaction was terminated after a final extension at 72°C for 10 minutes,

PCR to generate 253-bp product

A 253-bp fragment comprising of RRDR was amplified by PCR. The PCR mixture (20 ul) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl, 5 percent DMSO, 20 uM 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 synthesised at R & D Systems Europe Ltd. UK) and 1 unit of Taq polymerase (Bio line). The reaction mixture was denatured at 93°C for 2 minutes followed by 35 cycles each of denaturation al 93°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. The reaction was terminated after a final extension at 72°C for 10 minutes. The products were checked on 2 percent agarose gel electroplioresis usin g the standard procedures.

Separation of DNA strands

1. Heat denaturation: To 10 ul of the nested PCR product, 5 ul of loading solution (0.25% bromophenol blue, 0.25% xylene cyanol in 95% formamide) was added and heated at 95°C for 5 minutes. The contents were cooled in ice for 3 minutes before loading immediately on to the gel.

2. Alkali denaturation: To 10 ul of the nested PCR product, 2 ul of 0.5 M NaOH-10 mM EDTA solution was added and heated at 45°C for 5 minutes. The contents were cooled and loaded as above.

3. Separation of biotinylated strand by streptavidin beads: The biotinylatcd PCR product was captured using streptavidin magnetic beads as per the instructions given by the manufacturer (Dynal UK). In brief, 40 ul of the beads, suspended in 2x binding and washing buffer (BW buffer; 10 mM Tris, 1 mM EDTA and 2 M NaCl), was mixed with 20 ul of PCR product and made upto 80 ul. The mixture was incubated at room temperature (RT) for 30 minutes. After washing with 1x BW buffer, the captured DNA was denatured by adding 8 ul of 0.1 M NaOH and incubating for 10 minutes at RT. The alkali containing the denatured unbiotinylatcd strand (UBS) was aspirated, made upto a volume of 50 ul in sterile distilled water and then precipitated by the standard ethanol-sodium acetate method. The precipitate was resuspended in 5 ul of 1x TE buffer. The beads with the captured biotinylated strand (BS), were treated with 50 ul of 0.1 M NaOH and washed a further 3 times with 1x BW buffer and finally suspended in 5 ul of 1X TE buffer. The separated strands were heated at 95°C for 5 minutes with an equal volume of loading buffer and then cooled and loaded as above.

Polyacrylantide gel electrophoresis:

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 TBE buffer, 2.0 ml of glycerol, 21 ul of TEMED and 210 ul of 10% ammonium persulphate (Sambrook *et al.* 1989). The dimensions of the gel were 180 x 160 x 1.6 mm. The electrophoresis was carried out in Hoefer SE 600 vertical unit using 0.5x TBE buffer at 200 volts for 3 hours at RT. The separated strands were visualised by silver staining.

Silver Staining:

The DNA strands in the gel were stained with silver as described by Ainsworth *et al.* (1991). In brief, the gel was treated with ethanol (10%), Nitric acid (1%) and silver nitrate (0.2%)-formaldehyde (0.1%) solution, respectively for 5, 3 and 20 minutes. After washing 3 times with double distilled water the gel was treated with sodium carbonate (3%)-formaldehyde (0.025%) solution to develop the stain. The gel was preserved in 10% glacial acetic acid and photographed.

Criteria for rifampicin resistance:

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.

Results

Heat and alkali denaturation of PCR products for SSCP analysis proved unsuccessful in our hands for the generation of single stranded DNA products (Fig.). During SSCP analysis the single DNA strands were found to reanneal to yield double stranded product which was visualised after staining. On the other hand prior separation of the DNA strands using streptavidin magnetic beads consistently yielded good SSCP analysis. Treatment of DNA strand with S1 Nuclease prior to SSCP analysis confirmed the single-strand nature of the stained bands (results not shown).

The observation of the migration patterns of UBS and BS in 21 samples is given in the table. In 3 samples (No. 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.

Discussion

We experienced rapid reannealing of heat or alkali denatured DNA which prevented the separation of single-stranded products upon SSCP analysis. Factors that affect SSCP analysis were discussed by Yap and McGee (1994). One of the factors, that is the reannealing of the strands, especially when the product size is less than 100-bp, reduces the efficiency of SSCP analysis. 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 gel. Another factor could be due to large quantities of DNA (0.5 to 1.0 ug) applied to the gel for a strong signal after silver staining. A higher concentration of DNA is likely to promote rapid reannealing. Other detection methods, involving

radioactive labeling of PCR products, for example. may not encounter such a problem because less DNA is needed to generate a strong signal after SSCP. The use of radioactivity has its disadvantages, mostly in the hazardous nature of the use of radioisotopes. Yap and McGee (1991) had found 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 minutes before loading on to 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 the reannealing of strands. a novel approach was attempted. In this, a biotinylated primer was used in PCR. Then. the biotinylated and the unbiotinylated strands were separated and subjected to SSCP by PAGE. This approach also separates the two DNA strands in different lanes of the polyacrylamide gel, a process that is beneficial if the two strands have similar mobility that might confuse analysis. The recognition of shift in the migration of strands was thus made easy. In this study we proved an adaptation to the PCR-SSCP protocol which enables large amounts of DNA to be loaded onto the gel for subsequent silver stain detection and avoids the problem of single strand reannealing.

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 hands 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.* (1993a) observed a three-band pattern in his samples. In the present study, the occurence 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.* (1993a) observed a specific migration pattern for each of the nucleotide substitution.

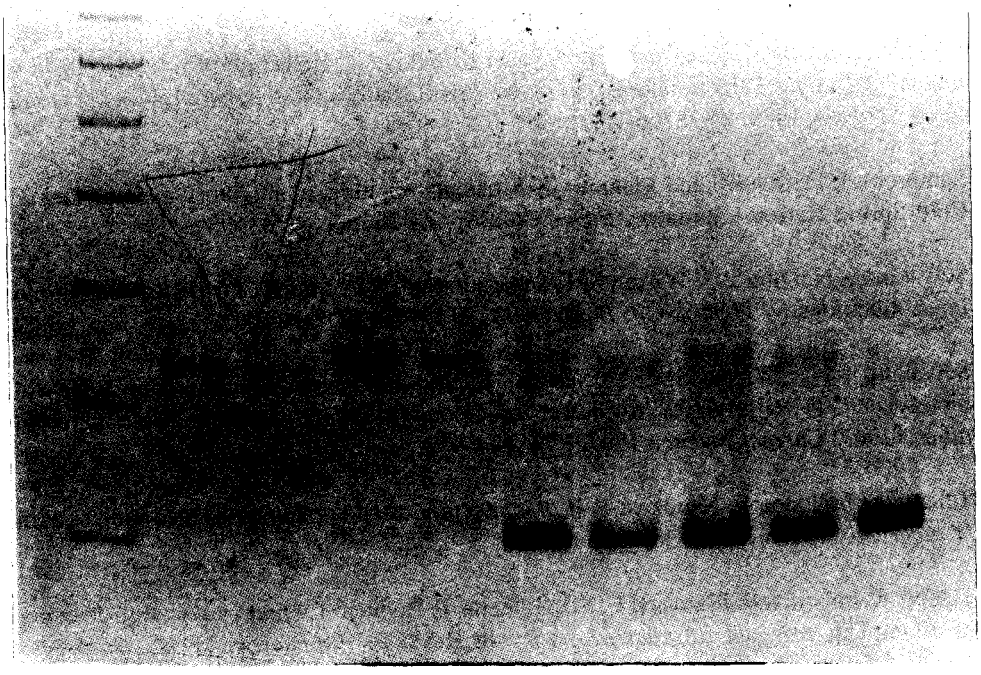
In this study 3 (No. 6, 20, 21) of 6 rifampicin sensitive strains were misclassified by the PCR-SSCP. It should be realised that SSCP does not differentiate rifampicin sensitive strains with functionally silent sequence changes. Therefore. DNA sequencing of the PCR products only could confirm the mutations 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, it was found that 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 automated PCR-SSCP (Telenti *et al.* 1993b) and automated sequencing (Kapur *et al.* 1994), 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. It is known that there are few mutations which could not be detected by any of the sophisticated molecular techniques. The results suggests that this procedure can be tried for the detection of such mutations in the RRDR of *M. tuberculosis.* In addition, a separate study using large number of sensitive and resistant strains need to be caked 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.

Table. The 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
1.	slow	slow	R	R
2.	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;
K: Resistant; S: Sensitive



Legend for the figure.

Lane
1. DNA marker
2. Biotinylated rif. resistant
3. Biotinylated rif. sensitive
4. Unbiotinylated rif. resistant
5. Unbiotinylated rif. sensitive
6. Alkali denatured rif. resistant
7. Alkali denatured rif. sensitive
8. Heat denatured rif. resistant
9. Heat denatured rif. sensitive
10. Undenatured double stranded DNA (Control).

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