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Single Strand Conformation Polymorphism (SSCP)-A REVIEW

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Abstract

SSCP (single strand conformation polymorphism) is a technique used for the detection of single-nucleotide polymorphism (SNPs). SSCP technique is a method capable of identifying most sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides in length. Current study deals about the basic principle, procedure, advantages, disadvantages and applications of SSCP in detail.

Key words- Polymorphism, Electrophoresis, PCR, SNPs, Nucleotide, Genotype, Mutation.

Introduction

Single-strand conformation polymorphism (SSCP), or single-strand *chain* polymorphism, is defined as conformational difference of single-stranded nucleotide sequences of identical length as induced by differences in the sequences under certain experimental conditions. This property allows to distinguish the sequences by means of gel electrophoresis, which separates the different conformations. The mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. The mobility of single strands, however, is noticeably affected by very small changes in sequence, possibly one changed nucleotide out of several hundred. Small changes are noticeable because of the relatively unstable nature of single-stranded DNA; in the absence of a complementary strand, the single strand may experience intrastrand base pairing, resulting in loops and folds that give the single strand a unique 3D structure, regardless of its length. A single nucleotide change could dramatically affect the strand's mobility through a gel by altering the intrastrand base pairing and its resulting 3D conformation.

Single-strand conformation polymorphism analysis takes advantage of this quality of single-stranded DNA. First announced in 1989 as a new means of detecting DNA polymorphisms, or sequence variations, SSCP analysis offers an inexpensive, convenient, and sensitive method for determining genetic variation (Sunnucks et al., 2000).

Like restriction fragment length polymorphisms (RFLPs), SSCPs are allelic variants of inherited, genetic traits that can be used as genetic markers. Unlike RFLP analysis, however, SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments (Orita et al., 1989). As a mutation scanning technique, though, SSCP is more often used to analyze the polymorphisms at single loci, especially when used for medical diagnoses (Sunnucks et al., 2000).

Work done in india and abroad using sscp (review of literature)

According to Hayashi,K.(2014),one advantage of PCR-SSCP OVER other methods of PCR based techniques in mutation detection is its simplicity

Xu *et al.* (2010) concluded that the PCR-SSCP assay demonstrates a high level of overall accuracy for the detection of rifampin resistance, which is a proxy for MDR-TB. This suggests that it has good utility as a rapid screening tool, especially in settings with high rates of MDR-TB. Use of the PCR-SSCP assay is currently limited to culture isolates and direct testing of smear-positive sputum specimens, and it is not commercially available. The PCR-SSCP assay is not recommended as a replacement for conventional culture and drug susceptibility testing for the detection

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of rifampicin resistance in *M. tuberculosis*.

Kalvatchev and Draganov, (2005) found when *SSCP* analysis was applied for investigation of variety viruses among lentiviruses, *Retroviridae*. Two hypervariable regions (in the *gag* gene and part of the V3 loop) are mainly analyzed in human immunodeficiency virus type 1 (HIV-1). By comparing of random paired matching of *SSCP* patterns between HIV-1 RNA and provirus DNA was found that the HIV-1 *env* target region diverged in 18 of 21 instances. The generation of characteristic *SSCP* patterns enables the rapid differentiation of bovine immunodeficiency virus (BIV) variants derived from the conserved part on the *env* region of the BIV genome, reducing the need for expensive and time-consuming direct sequencing analyses. The results demonstrated genetic polymorphism among a number of sampled BIV population in experimentally infected rabbits. At least three *SSCP* patterns (BIV quasispecies) are detected not only between different animals, but also between different organs in the same animal. An analysis of the genetic variability in the nonstructural gene of human parvovirus B19 by *SSCP* revealed the presence of six genotypes among 50 samples of virus from several countries. Sequencing of this region confirmed the presence of mutations in the different genotypes, and all are silent mutations. There was a good correlation between the *SSCP* type and the country from which the virus was obtained .

Humphries *et al*.(1997) said that the SSCP technique is a method capable of identifying most sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides in length. Under nondenaturing conditions a single strand of DNA will adopt a conformation (presumably dependent on internal base-pairing between short segments by foldback) that is uniquely dependent on its sequence composition. This conformation will usually be different if even a single base is changed. Most conformations seem to alter the physical configuration or size sufficiently that, even though the variant sequence has the same charge, the configuration-to-charge (size-to-charge) ratio is different enough to be detectable as a mobility difference upon electrophoresis through a retarding matrix such as acrylamide gel. Typically, the duplexes will be from the same PCR reaction for samples with possible genotypic differences. In many laboratories [α-^{32}P]dCTP is incorporated during the PCR, and diluted PCR product is denatured by a brief boiling step, after which the sample is loaded on a nondenaturing “sequencing” (40 cm) acrylamide gel. The samples thus adopt a single-stranded secondary structure because of the formation of interstrand base pairing. However, although the samples have been diluted, they concentrate upon entry into the gel, and this allows some reannealing to take place. The considerations implicit in classical “Cot” studies of reannealing (concentration of each single strand, DNA complexity, and time) apply . Thus, to reduce the proportions of PCR product that reform as a double strand and reduce the amount available as single strand for polymorphism analysis, the sample needs to be relatively dilute. When using thin gels with small capacity for sample volume, this concentration consideration may limit the choice of DNA detection methods, with radioisotope detection being the method of choice because it is considerably more sensitive than ethidium bromide or even silver-staining methods

Kubo *et al*.(2009) SSCP is also widely used in virology to detect variations in different strains of a virus, the idea being that a particular virus particle present in both strains will have undergone changes due to mutation, and that these changes will cause the two particles to assume different conformations and, thus, be differentiable on an SSCP gel.

Toll-like receptor 4 (TLR4) sequence of Indian buffalo population was analysed for the detection of variation within exon2 region. 81 DNA samples representing six different buffalo breeds viz. Jaffarabadi, Marathwada, Toda, Pandharpuri, Mehsana and Chilika are amplified using the primers specific to exon 2 and adjoining intronic sequence. PCR products when resolved on non-denaturing polyacrylamide gel electrophoresis (PAGE) yielded two different variants A and B with almost equal frequency of 0.531 and 0.469, respectively, which further varied among the buffalo breeds investigated. Sequencing analysis of buffalo TLR 4 exon 2 revealed 167 nucleotides, coding for 55 amino acids as in other livestock species. Cattle and buffalo had 100% amino acid homology with few changes as compared to sheep and goat. The variants reported in the present study can be used as candidate markers for economically important traits in buffalo.

Gupta *et al.* (2005) reported that SSCP (single strand conformation polymorphism) could be used as technique for the detection of single-nucleotide polymorphism (SNPs)

Liu *et al*. (2011) reported genetic variations at exon 2 of TLR4 gene in 14 chicken breeds and the Red jungle fowl are detected by PCR-SSCP method and two alleles and three genotypes are found, Tibetan chicken and Red jungle fowl only had BB genotype, while the others had three genotypes of AA, BB and AB. The study demonstrated that there are differences of normal anti-disease ability in Chinese indigenous chicken breeds and showed no significant correlation with body size, product type and geographical location

Oraon (2013) subjected the PCR products obtained after amplification of sheep DNA with specific primers for FecB gene, to polyacrilamide gel electrophoresis for

detection of Single Strand Conformational Polymorphism (SSCP).The primers of *FecB* gene used by him are as follows. With the primer having forward and reverse base sequence as CCAGAGGACAATAGCAAAGCAAA and CAAGATGTTTTCATGCCTCATCAACAGGTC, respectively, six different SSCP variants are found which are located in two locus i.e Locus-1 and Locus-2 *FecB* genes. Locus-1 with genotype AA, AB and BB and at Locus-2 with genotype CC, CD and DD of *FecB* gene was found.

SSCP is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility through a gel.

SSCP Procedure

The procedure used during the development of SSCP is as follows:

- digestion of genomic DNA with restriction endonucleases
- denaturation in an alkaline (basic) solution
- electrophoresis on a neutral polyacrylamide gel
- transfer to a nylon membrane
- hybridization with either DNA fragments or more clearly with RNA copies synthesized on each strand as probes (Orita et al., 1989).

Since then, more convenient procedures have been developed, taking into account other molecular techniques, although sometimes it is simpler to amplify the double strand and then denature it into single strands instead of trying to find suitable primers for the below PCR method if the targeted sequence is unknown. The procedure used by Kumar (2013), Murmu(2011) and Oraon(2013) is being mentioned in detail as follows.

Standard Protocol of SSCP

1. Collection of blood samples

Blood samples are collected in vaccutainers. An anticoagulant (EDTA) is mixed in blood. The blood samples are brought in ice as coolant to maintain low temperature.

2. Isolation of Genomic DNA

Genomic DNA is isolated and purified from white blood cells using proteinase K digestion and standard phenol: chloroform extraction as per the standard protocol described by Sambrook *et al.* (1989).

The detailed protocol for isolation of DNA is as under

1. Two volume of ice-cold lysis buffer is added to whole blood and mixed well by vortexing. The tube is kept on ice for 10 min., centrifuged at 10,000 rpm for 10 min. at 4 °C to pellet WBC. The supernatant is discarded carefully.
2. Above step is repeated until most RBC are lysed and clear pellet of WBC is obtained (2-3 ishes are sufficient to achieve clear WBC pellet).
3. Five ml digestion buffer (annexure II) is then added to the WBC pellet and mixed well by vortexing.
4. 20% sodium dodecyl sulphate is added so that its final concentration becomes 0.5%, followed by addition of 100µg proteinase K, mixed well and tube is incubated at 56°C for overnight in a water bath.
5. After overnight incubation, equal volume of equilibrated phenol is added and mixed thoroughly to form uniform suspension and centrifuge at 12,000 rpm for 10min at room temperature.
6. Upper aqueous phase (containing DNA) is collected in a separate oakridge tube.
7. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24:1) is added and mixed well to form uniform suspension. The suspension is centrifuged at 12,000 rpm for 10 min at 25°C.
8. Upper aqueous phase is saved without disturbing the interphase.
9. Equal volume of chloroform: isoamyl alcohol (24:1) is added and mixed properly.
10. Upper aqueous phase is retained and DNA is precipitated by adding 0.1 volume sodium acetate (pH 5.5) and 2 to 2.5 volume of chilled ethanol.
11. Precipitated DNA is spooled, ished with 70% ethanol and dried.
12. Extracted DNA is dissolved in TE buffer (pH 8.0) and stored at 4°C.
13. DNA is incubated in TE at 56°C for 30 min. for complete solubilization.

4.Storage of DNA

20 µl of stock solution of DNA is diluted serially to a concentration of 50-100ng/µl as working stock for further analysis. Rest of the stock solution of DNA is stored at -20^{o}C.

5. Quality and quantity check

Quality and quantity check of isolated genomic DNA sample is done by agarose gel electrophoresis. Agarose gel electrophoresis method is used for separating and analyzing charged biomolecules like DNA, RNA and proteins. The use of gels such as starch, polyacrylamide and agarose as supporting media provided enhanced resolution particularly for nucleic acids and proteins. The location of DNA within the gel is easily detected by staining with ethidium bromide and very small quantities of DNA (1-10ng) is detected by this method. Small sized DNA fragment pass through smaller pores in the gel and the larger fragments pass through larger pores. Larger sized DNA molecules face a large frictional force as they move through the gel and so have lower mobility as compared to the smaller sized DNA fragments that experienced smaller force of attraction. A mixture of DNA molecules, therefore, separated into discrete bands during electrophoresis. Frozen stock of genomic DNA samples are diluted to1:10 and their quantity and quality is assessed by running in 0.6 % agarose gel using 1X TAE buffer and also with Spectrophotometer (Nanodrop). After checking, the selected samples are adjusted to a concentration of 50-100ng/µl for further processing.

The complete protocol of agarose gel electrophoresis {as done}is presented below:

6. Preparation of agarose gel

1. 2ml of 50x TAE is diluted to make to 100ml in a 250ml Erlenmeyer flask to give final concentration 1x.
2. The required amount of agarose powder is weighed and added to the 1x TAE buffer solutions.
3. The slurry is heated in a microwave oven until agarose is completely dissolved. In microwave oven, slurry is heated for 3 min in 1 min increments, swirling the solution gently between heating cycles to release trapped air and re-suspend any agarose particles caught on the side of the flask.
4. The agarose solution is cooled until it reached a temperature of approx. 50-55°C. The flask is swirled occasionally to keep the contents at uniform temperature and prevent the agarose from gelling at the bottom of the flask. Swirling is done gently, to prevent the formation of bubbles, Ethidium bromide solution (from a stock solution of 10mg/ml in water) is then added so that its final concentration became 0.5µg/ml.

7. Gel casting

1. The gel tray (gel casting plate) is placed on horizontal surface (checked with a level).
2. The comb is placed at the desired position on the gel tray 0.5-1.0 mm above the surface of the gel plate so that the complete well is formed. The teeth is prevented from touching the gel bed; checked by pushing a folded paper towel beneath the teeth.
3. The warm agarose solution is poured into the mold until its thickness became 3 mm, care is taken to avoid the air bubbles under or between the teeth of comb.
4. After the gel is completely set (40 minutes at room temperature), the comb is removed carefully by wriggling back and forth gently and then lifting up.

8. Loading the gel

The gel along with gel tray is placed in the electrophoresis tank and the top of the gel is flooded with fresh running buffer (1xTAE) to cover the gel to a depth of about 1-2mm. The DNA samples are mixed with desired gel loading buffer and loaded into wells. A piece of dark paper is placed beneath the gel tank to make the wells more visible. Also care is taken not to perforate the bottom of the wells while loading the samples. The gel loading buffers served three purposes:

(i) they increased the density of the samples,
(ii) Ensuring that the DNA dropped evenly into the wells,
(iii) They added color to the samples, thereby simplifying the loading process, and they contained dyes that in an electric field moved towards the anode at predictable rates. About 2µl of dye and 10 µl of amplified DNA is loaded in each well. A voltage of 80 volts is applied. The gel is run until the Bromophenol blue and Xylene cyanol FF had migrated the appropriate distance through the gel. The gel is run for about seven hours in each case.

9. Visualization of DNA

On completion of electrophoresis, the gel is visualized under UV transilluminator (Bio-Rad) and quality and quantity is judged. DNA appeared as single compact pink fluorescent

band free from degradation (whole lane fluorescent) or RNA contamination (fluorescent spot migrated away from well).

10. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a relatively simple but powerful technique that amplifies a DNA template to produce multiple copies of specific DNA fragment in vitro.

11. Optimization of PCR Reactions

The PCR reaction is optimized to attain the required optimum temperature and time range to attain maximum amplification for each DNA sample. The details of PCR running condition is presented in table1

Table 1.Final PCR programme for PCR amplification, (Kumari,2010)

S. No.	Reaction	Temperature	Time	
1	Initial denaturation	94^0C	3min	
2	Denaturation	94^0C	30 sec	33 cycles
	Annealing	58.5^0C	30 sec	
	Extension	72^{0C}	30 sec	
3	Final extension	72^0C	7 min	
4	Holding temperature	4^0C		

12. Parameters and optimum condition for PCR

When setting up PCRs for first time with new template DNA, new primers, or a new preparation of thermo stable DNA polymerase enzyme, amplification is generally less than optimal.

Optimization and standardization of PCR protocol is usually required to suppress non specific amplification and to enhance the yield of the desired PCR product. The details of Parameters and optimum condition for PCR running is presented in table 2.

13. Agarose gel electrophoresis of PCR amplified DNA

Before running in PAGE, the 2 µl of PCR products are checked in 2% agarose for the amplification. Before loading into the wells, gel loading dye (bromophenol blue in glycerol) is added to the sample and the samples are run under constant voltage condition (80V) till the two dyes got separated. Amplified products appeared as sharp orange color bands under UV Transilluminator due to the intercalation of ethidium bromide .

Table 2. Parameters and optimum condition for PCR,(Kumari,2010)

The PCR-SSCP analysis of TLR -1 gene by Kumar

PCR paramaters	Tested range	Optimum condition	Remarks
Genomic DNA concentration(50-100µg/µl)	2.5µl,1.5µl,1µl,2µl	1.5µl	Absence of amplification with lower concentration.
Magnesium chloride(25mM)	0.5µl 1µl,1.5µl	0.5µl	Excess/lower concentration increases the non- specificity and yield of the product.
Deoxynucleotide triphosphate (DNTPs in 10mM) (SIGMA)	0.5µl 1µl,1.5µl	0.5µl	Increased concentration reduces the free Mg^{++} interfering with the enzyme.
Primer concentration(µM) Forward (20ng/ µl) Reverse (20ng/ µl)	0.5µl 1µl,1.5µl each	0.5µl each	Lower & higher concentration leads to absence of amplification and primer-dimer formation
Taq polymerase(5units/µl) (SIGMA)	0.3µl 0.4µl,0.5µl	0.5µl	Lower concentration showed proper amplification. High concentration showed decreased specificity.
10X PCR buffer	0.5µl 1µl,1.5µl	1.5µl	Lower concentration showed improper amplification.
Autoclaved Milipore Water		4.5 µl	
Total		10 µl	

(2013) revealed the polymorphic pattern of genotypes in Swine. He further mentioned that Single strand conformational polymorphism is a tool for mutation detection at DNA level. It is a conformation based scanning method of polymorphism detection which is simple and widely used method. In SSCP the target region is amplified by PCR and then amplified region is denatured to generate single stranded DNA and separated by electrophoresis on a non denaturing polyacrylamide gel. The single stranded fragments adopt three dimensional conformations, which is dependent on the primary sequence. If sequence differences exists between wild type and mutated type DNA, this may result in a mobility shift observed during gel electrophoresis because the conformation of single strand DNA is also affected largely by electrophoretic condition, optimal condition are determined empirically which limit the standardization procedure.

To explore genetic polymorphism in TLR1gene, amplified PCR products are subjected for SSCP through polyacralamide gel electrophoresis.

14. Polyacrylamide gel electrophoresis (PAGE)

Glass plates and spacer combs are washed with detergent, ringed under running tap and finally in double distilled water before drying. Before use glass plates, spacer and comb are wiped with ethyl alcohol.

Glass plates are fitted by putting the 1mm spacer between the two plates at its position and then clamp is applied. Flat end of glass plates is sealed with adhesive tape. Side end get sealed automatically. Then the gel mix is poured into the space between the plates and finally comb is inserted immediately. The gel is allowed to polymerize at room temperature. After polymerization, adhesive tape is removed from the sealed end. Comb is removed and then flushed the wells with double distilled water. The gel is put in to electrophoresis tank with notched plate facing towards the buffer reservoir. The reservoir is filled with 1X TBE. The gel is given pre run at 250 V for 30 min to remove any polar impurity, in the vertical gel electrophoresis system.

Table 3.Composition of polyacrylamide gel,(Kumar,2013)

Reagent	Quantity in 30 ml mixture
Acrylamide : Bisacrylamide (39:1) Stock solution 30%	9ml
10 X TBE	3ml
Ammonium per sulphate (10%)	300µl
TEMED	30µl
Poly ethelene Glycol (5g/lit) MW 4000 (Markoff *et al*.,1997)	300µl
Mili Q Water	17.37 ml

PCR product (amplicon) is mixed with formamide dye, mixed is then loaded in gel with the help of long tip micropipette carefully. Electrophoresis is performed at 4^0 C temperatures for 6 hours at 200 V. After running is over, gel is kept for silver staining to visualize the banding pattern.

15. Silver staining

Silver staining method is described by Bassam *et al.* (1991) and it is highly efficient. It can detect nucleic acid in the pictogram range by the specific chemical reduction of silver ions. Silver binds to nucleic acid bases and is then selectively reduced by chemical reagent. The presence of formaldehyde in the silver stain improves both sensitivity and contrast. Formaldehyde selectively reduces silver ions to metallic silver. Thiosulphates helps in dissolving insoluble silver salts by complex formation removing silver ions from the gel surface. The presence of thiosulphate eliminates the formation of dark precipitate in the gel and developer solution.

Procedure of silver staining

1. Silver staining is carried out according to Bassam *et al.* (1991) with few modifications.
2. The gel is placed on clean surface by keeping the notched plate facing upwards
3. The gel along with the plate is placed in a suitable sized tray.
4. 100 ml of ethyl alcohol is added in to 900 ml of ultra pure water. Tray is shaken gently and the plate is removed carefully. The gel is agitated slowly for 30 min with the help of gel rocker till the dye is no longer visible.
5. The gel is ringed 3 times for 2 min each with double distilled water.
6. 0.7 % (2.1 ml of conc. Nitric Acid in 300 ml of distilled water) Nitric Acid solution is added in gel and agitated for 6 minute on gel rocker.
7. Again the gel is ringed 3 times for 2 min each with double distilled water.

8. Freshly prepared staining solution containing 250 ml of pure water, 250 mg silver nitrate and 370µl formaldehyde is used. Solution is added to tray and agitated slowly for 30 min.
9. The gel is washed with double distilled water within 20 second.
10. For developing the gel 500 ml developing solution is prepared. Solution contains 500 ml double distilled water, 15 gm sodium carbonate and 750µl formaldehyde. Solution is then poured in tray and gel is gently shaken till developing of bands.
11. The gel is fixed in 10% ethyl alcohol solution. Finally gel is washed with double distilled water for 2 min. and then dried in gel dryer.
12. The gel is visualized under light and documented.

Applications

1. Ensuring that intraspecific variation is adequately represented in phylogenetic sequencing projects.
2. Screening large population samples for mitochondrial DNA sequence variation.
3. Developing and screening sequence-variable markers.
4. Getting the most out of microsatellites: length homoplasy and sequence variation.
5. Investigating complex mixtures of similar length equences: pseudogenes and multigene families
6. SSCP is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility through a gel.
7. SSCP analysis detects sequence variations (single-point mutations and other small-scale changes) through electrophoretic mobility differences. These variations can potentially cause conformational changes in the DNA molecules.
8. Under nondenaturing conditions and often reduced temperature, single-stranded DNA molecules can assume unique conformations that vary depending on their nucleotide sequences. These conformational changes can result in detectable differences in mobility.
9. The difference in shape between two single-stranded DNA strands with different sequences can cause them to migrate differently on an electrophoresis gel, even though the number of nucleotides is the same, which is, in fact, an application of SSCP.

SSCP Limitations and Considerations

1. Single-stranded DNA mobilities are dependent on temperature. For best results, gel electrophoresis must be run in a constant temperature.
2. Sensitivity of SSCP is affected by pH. Double-stranded DNA fragments are usually denatured by exposure to basic conditions: a high pH. Kukita et al. found that adding glycerol to the polyacrylamide gel lowers the pH of the electrophoresis buffer--more specifically, the Tris-borate buffer--and the result is increased SSCP sensitivity and clearer data.
3. Fragment length also affects SSCP analysis. For optimal results, DNA fragment size should fall within the range of 150 to 300 bp, although SSCP analysis of RNA allows for a larger fragment size (Wagner, 2002). The presence of glycerol in the gel may also allow a larger DNA fragment size at acceptable sensitivity (Kukita et al., 1997).
4. Under optimal conditions, approximately 80 to 90% of the potential base exchanges are detectable by SSCP (Wagner, 2002).
5. If the specific nucleotide responsible for the mobility difference is known, a similar technique called Single Nucleotide Polymorphism (SNP) may be applied.

Conclusion

SSCP is one of the many sensitive techniques available today for assaying sequence variation .All of them have their advantages and disadvantages in terms of sensitivity, optimization, cost and requirements for equipment. Overall, we find that SSCP presents a very favourable combination of characteristics that is not found in any other techniques.It can be concluded that SSCP is one of the best and cost effective method to detect DNA polymorphism and mutation.

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