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Applications of polymerase chain reaction: Single strand conformation polymorphism to diagnose disease causing microorganisms

S.Umesha*, S.Chandrashekar, S.Chandan

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore - 570 006, Karnataka, (INDIA)

E-mail : umeshgroup@yahoo.co.in

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ABSTRACT

Diagnosis of disease causing microorganisms is still primarily based on conventional methods *viz*., staining, cultures and biochemical analyses etc., A series of molecular techniques such as Polymerase Chain Reaction-Single Strand Conformation polymorphism (PCR-SSCP), Random Amplified polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) etc., are increasingly introduced and incorporated in the clinical microbiology laboratories world wide. These techniques are, rapid and offer high sensitivities, screening method which is cost-effective and the entire procedure takes around 6 to 7 h only, and hundreds of samples can be screened at a time. We have attempted to consolidate the literature about the utilization of PCR-SSCP technique in diagnosing the disease causing microorganisms.

KEYWORDS

Application of PCR-SSCP;
Microorganism;
16S rRNA gene;
Non-denaturing PAGE.

INTRODUCTION

Single-strand conformation polymorphism analysis is a rapid and sensitive approach for characterizing DNA sequences. This method was first described by Orita et al.[28,29], and SSCP has since been successfully used for detecting various alterations in DNA base sequences, including substitutions, deletions, insertions, and rearrangements. Polymerase chain reaction (PCR) products are now routinely used for SSCP analysis (PCR-SSCP)[6]. After PCR amplification of the target sequence, the amplified product is denatured to single stranded DNAs (ssDNA) and subjected to non-denaturing polyacrylamide gel electrophoresis. Under non-denaturing conditions, ssDNA has a secondary structure that is determined by the nucleotide sequence. The mobility of the ssDNA depends on the secondary structure of the amplified product. Bands of ssDNA at different positions on the gel indicate different sequences. PCR-SSCP is capable of detecting >90% of all single-base substitutions in 200bp fragments[19] make it a method of choice for screening DNA fragments in many research and diagnostic applications. Progress in molecular biology brought a lot of information about DNA sequences. Using different techniques of DNA analysis in combination with published existent data, it is possible to identity variability within and between populations[1]. Some of the methodologies more frequently used

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for the identification of point mutations are DGGE (Denaturing Gradient Gel electrophoresis)[7], TGGE (Temperature Gradient Gel electrophoresis)[30], Ribonuclease[24] and Chemical[3]. The application of PCR-SSCP technique to molecular diagnostics holds great promise for the early detection of clinically important microorganism because of its technical simplicity and relatively high sensitivity for the detection of sequence variations.

PRINCIPLE

Electrophoretic mobility of a particle in a gel is sensitive to both its size and shape. In non-denaturing conditions, single stranded DNA has a folded structure that is determined by intramolecular interactions, and therefore by its sequence. In SSCP analysis, a mutated sequence is detected as a change of mobility in polyacrylamide gel electrophoresis caused by its altered folded structure as in described fragments of cloned mutated F_1-ATPase gene of *Esherichia coli* that move anomalously in strand separated gel electrophoresis[15,23]. It may be seen that only limited sequence changes can cause detectable structural change of the molecule. However, it was found that because of its high resolving power, polyacryalimde gel electrophoresis can distinguish most conformational changes caused by subtle sequence differences such as one base substitution in a several hundred base fragments. At present, it is not possible to predict the shift of electrophoretic mobility induced by the mutation. Conversely measurement of the mobility of directionally mutated sequences may provide an empherical approach to the prediction of higher order structure of single-stranded nucleic acids[19].

SENSITIVITY

Much of the literature on SSCP deals with factors affecting sensitivity to detect single-base changes. Since, reported results are always particular to specific fragments and sequence changes. Mutations that show no mobility shift under one set of conditions may be revealed under different conditions. Concentration ranges of acrylamide (usually from 4% to 12%) and cross-linker bis-acrylamide (usually from 2% to 3.4%) of the concentration of acrylamide have been reported to be beneficial in particular circumstances[10,13,32] as have additives such as 5-10% glycerol, 5% urea or formamide and 10% dimethylsulfoxide or sucrose[10,28,29]. Alterations in gel running temperature from 4°C to 37°C and alkaline pH of buffer leading to changes in gel may also help. Purine-rich strands may be more sensitive to base changes than pyrimidine-rich strands[10]. Smaller fragments (<300 bp) are in general, more likely to reveal single-base changes[12,13,35] although fragment size and sequence context (the sequence of adjacent DNA) can have unpredictable effects on mobility shifts associated with particular base changes[5]. At high concentration, denatured PCR products may re-anneal quickly, there by complicating or preventing SSCP analysis; this may be prevented by including 33mM methyl mercury (II) hydroxide in the denatured sample buffer or by adding a stacking gel containing 75% formamide to keep complementary strands denatured until they separate in the resolving gel[36,39,41].

PRACTICE

SSCP analysis is generally considered to be the most suitable technique for the detection of mutations in short stretches of DNA. Hence, the size of PCR fragments investigated is usually in the range of 175-250 bp. It is important to optimize the PCR reaction to minimize unwanted products which may interfere with gel analysis. The PCR products should be evaluated for purity by agarose gel electrophoresis before being loaded onto a SSCP gel.

Sample preparation

5µl of individual PCR products were mixed with 25µl of the denaturing buffer (95% formamide, 20mM EDTA, and 0.05% bromophenol blue). The mixtures were heated at 96°C for 10 min and then chilled on ice[19].

Nondenaturing polyacrylamide gel electrophoresis

Denatured PCR products were loaded on an 8% acrylamide-bisacrylamide non denaturing gel with 8ml of 40% acrylamide/bis, 4 ml of 10x TBE, 40µl of TEMED, 400µl of 10% APS and 28ml of water. APS were added as polymerization catalysis to 40ml gel mix.

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The gel was casted using the gel sandwich set[19]. Thirty microliter of each mixture was loaded, an aliquot of 25ng of single-stranded DNA (ssDNA) ladder was also loaded in both left and right lanes of a gel to facilitate comparison of SSCP patterns[18]. Denatured PCR products were electrophoresed in prechilled 1x TBE buffer (Tris borate 89mM, Boric acid 89mM, 20mM EDTA, pH 8.0) at 200v for 2 h at room temperature. After electrophoresis, polyacryalimde gels were peeled from the glass plates and silver staining was carried out[19].

Silver staining: The gels were soaked in 50 ml of 10% ethanol for 10 min, and placed in the same amount of 1% nitric acid for 3 min. After two brief washes with 100 ml distilled water, gels were stained in 50 ml of 2ppm silver nitrate (made from 100x stock stored at 4°C) for 20 min then rinsed three times in 200 ml of distilled water. Gels were developed by brief rinsing in 30 ml of 1ppm formaldehyde in 3% sodium carbonate until desired band intensity was reached. The stain was fixed in 1% acetic acid, once the SSCP patterns were visible, images were captured for documentation and comparison analysis between species SSCP banding of individual isolates were analyzed with the aid of the ssDNA ladder[19].

APPLICATIONS OF PCR-SSCP TO DETECT MICROORGANISMS

Candidatus phytoplasma detection using PCR-SSCP

Schneider et al.[33] employed the SSCP for the differentiation and molecular characterization of *Candidatus* phytoplasma mali, the causative agent of apple proliferation in orchards across Europe. Earlier attempts for characterization using 16S rRNA sequences failed because the sequence was found to be identical in most of the strains and, it has also been demonstrated that the closely related pathogens *C.*phytoplasma mali, *C.* phytoplasma pyri and *C.* phytoplasma prunorum, (all members of the apple proliferation phytoplasma group) display more than 97.5% homologous 16S rRNA sequences. This study employed the molecular characterization of a marker linked to virulence that could be used as a basis for strain differentiation. A 530 bp fragment of two genes, ATP00034 and ATP00464 from hflB gene was chosen for analysis by SSCP. The study was conducted using infected plant material obtained from different sources in Europe. Sequencing of the 530 bp fragment obtained from SSCP for each of the samples was done and its subsequent analysis showed that the *C.* phytoplasma mali strains shared a nucleic acid homology ranging between 94.2% to 100%, with identical sequence homology noticed in strains that showed identical SSCP profiles. These strains also occupied identical positions in the phylogenetic tree. A higher level of homology was evident at the protein-level as most of the substitutions were silent mutations. The homology of nucleic acid sequence within the amplified region was seen to be ranged between 85.9-90.8% for *C.* phytoplasma pyri and *C.* phytoplasma mali, and 84.6-87.8% for *C.* phytoplasma prunorum, and *C.* phytoplasma mali respectively. Thus, the SSCP analysis of the *hfl*B gene was demonstrated to be effective for typing *C.* phytoplasma mali strains.

Trichinella populations studies using PCR-SSCP

Gasser et al.[9] analyzed the sequence variability in the Expansion segment (ES5) and domain IV and the D3 domain of nuclear ribosomal DNA within and among isolates of *Trichinella* genotypes using cold SSCP. Investigating the genetic make up of *Trichinella* populations is important in transmission patterns and control. Various DNA based techniques such as DNA-hybridization and sequencing of genes of the nuclear or mitochondrial genomes are useful in identification of some species and genotypes for genetic variation within the genus. However, PCR has the ability to specifically amplify sequence from individual, *Trichinella* larvae, using multiplex PCR of the ES5 within the domain IV and ITS of nuclear ribosomal DNA (r DNA) allows the identification of species and genotypes of *Trichinella* based on the size and specific amplicons in agarose gels, but fails to detect sequence variation with isolates and within or among individuals. To overcome these problems, SSCP approach has been used. SSCP analysis showed variability of two nucleotides in the ES5 regions among *T. spiralis* samples, whereas, no sequence heterogeneity was detectable in D3 domain by SSCP or sequencing within or between isolates of *T. spirales*, and SSCP also distinguished *T. mativa* from *Trichinella* T6 in ES5 region but, failed to distinguish among them in D3 region. So, in this study using PCR-

SSCP, genetic make up of *Trichinella* populations is identified.

Bacterial identification

Oh et al.[26] employed Capillary electrophoresis based single-strand conformation polymorphism (CE-SSCP) to detect pathogens that are associated in food poisoning. Earlier attempts of detection were direct plating and biochemical tests. These methods required separate cultivation of each strain, which is time consuming and labour intensive. In this study, using 16S rRNA , gene-specific PCR Capillary electrophoresis Single-strand conformation polymorphism electrogram detected five pathogens *viz., Esherichi coli, Clostridium perfringes,Campylobacter jejuni, Salmonella enteric and Bacillus cerus* that were differentiated by electrograms of each strain in which one major peak and several minor peaks were observed, thus differentiating these pathogens individually. Oh et al.[26] concluded that, CE-SSCP offers rapid and effective identification of the food borne pathogens.

Nair et al.[25] employed SSCP for the differentiation and molecular characterization of *Salmonella*, the causative agent of enteric fever and gasteroenterities in human beings. Earlier attempts used to detect *Salmonella* serovars was done by phenotypic methods, serotyping and phage typing using molecular techniques such as pulse field gel electrophoresis, PCR-Ribotyping, RAPD and RFLP etc., However, these techniques are laborious and time consuming and they failed to discriminate at serovars level *i.e*., at the intraserovar and interserovar. Therefore in this study, PCR-SSCP and PCR-RFLP were used to detect polymorphisms within the *groEL* gene which encodes a heat shock protein (groEL) - a member of the stress response protein (HSP60) family. Forty one strains with ten different *Salmonella* serovars were studied using 1.6kb *groEL* gene. The data generated by PCR-RFLP using *HaeIII* restriction sites within *groEL* genes confirmed that, there was a difference in *Salmonella* serovars which generates three distinct profiles. *S. enterica* serovars typhimurium and serovar virchow in profile 1, serovar hadar, serovar paratyphi A, and serovars paratyphi B in profile 2 and serovar arizona strain exhibited completely different RFLP profiles. However, by PCR-RFLP, there was no clear discrimination between the serovars and serogroups. Using *HaeIII* digested *groEL* product, PCR-SSCP was performed, as SSCP technique alone does not work efficiently for larger gene fragments. Among fourty one strains of ten different serovars *viz*., tryphimurim, newport infantis, hadar and virchow, produced fourteen different PCR-SSCP profiles, which discriminated each *Salmonella* serovars and serogroups. Therefore, in their study PCR-SSCP analysis was demonstrated as a rapid, simple and sensitive molecular technique in characterizing different *Salmonella* serovars.

Gillman et al.[11] applied multiple-fluorescence-based PCR and subsequent SSCP analysis for species specific identification of mycobacteria. The species level identification of mycobacteria is done by culture and biochemical testing methods, which are time-consuming. In order to increase the speed and specificity of identification, the technique of multiple fluorescence based SSCP analysis was developed for this organism. Four variable regions of the 16S rRNA gene of the mycobacteria were chosen to design primers. Four sets of primers with different fluorescent labels were used in two different duplex PCR reactions for each of the species and also for test strains. SSCP electrophoresis was performed for each of both the PCR reactions. The SSCP pattern for each of the species was analyzed using the Bionumerics v. 1.5 software. The retention times of each of the fluorescence labeled PCR-fragments for each primer for each of the species were entered into the database. This dataset was then used to identify unknown SSCP patterns, by comparison of the retention time of the unknown against the database.

Windjojoatmodjo et al.[40] employed PCR-SSCP technique to identify the bacteria up to genus and species levels. Using two sets 16S rRNA conserved primers *viz.*, P11P-P13P and ER10-ER11, over hundred strains, spanning fifteen genera and forty species were examined using bacterial cell lysates. PCR products were analysed using SSCP and detected using silver staining in which species specific banding patterns were obtained for *Clostridium spp. Listeria spp. Pseudomonas spp* and *Enterobacter spp*.

Fungal identification

Diba et al.[4] employed PCR-SSCP technique for the differentiation and molecular characterization of medically important *Aspergillus* species. *Aspergillus* spe-

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cies are associated with allergic bronchopulmonary disease, mycotic keratits, otomycosis, nasal sinusitis and invasive infection. Earlier attempts for characterization were on the basis of macroscopic observation of development of fruiting structures. Diba et al.[4] used PCR-SSCP to identify medically important species from different clinical sources. PCR was performed using universal fungal primers ITS1 and ITS4, which amplified 350bp to compare and identify, which is more sensitive powerful in identifying the sequence polymorphisms. They used two methods, *i.e.*, PCR-RFLP and PCR-SSCP. PCR-RFLP by *MwoI* restriction enzyme characterized seven *Aspergillus* species *viz., A. flavus, A. tereus*, *A. nidulans*, *A. clavatus*, *A. ochraceus* and *A. amsteloidam*, but, RFLP is expensive and time consuming, therefore PCR-SSCP was used. Due to large fragments obtained by PCR, gradient polyacrylamide gel was used to detect sequence polymorphisms among *Aspergillus* species. Depending on the banding pattern obtained by PCR-SSCP, *Aspergillus* species has been differentiated into different sub-species *i.e., A. nidulans, A.fisheri, A.quadricincta* in one group and *A.flavus, A. tereus and A. ochraceus* in another group and PCR-SSCP discriminated these medically important *Aspergillus* species with less than 12 h and without the use of restriction enzymes.

Wang et al.[38] in their reports, have stated SSCP analysis of ribosomal DNA (rDNA), as a highly agile technique for the selective understanding of genetic variation (of, as little as one nucleotide difference in their rDNA) of phenotypcially similar yeast species. In order to the differentiating potential of SSCP, they comparatively studied different representative groups of yeast species (which were strongly linked with each other and had pretty much the same rDNA sequences) from ascomycetes and basidiomycetes. And their SSCP patterning results could neatly validate the identity of every different species. Thus, the study implies SSCP, as an efficient and economical tool to distinguish species-diversity amongst yeast species with different rDNA sequences in a large collection of yeast strains from natural sources.

Kong et al.[19] employed the PCR-SSCP technique for the differentiation and molecular characterization of *Phytopthora ramorum*, the causative agent of canker disease seen in many important forest trees such as Oak, Daughlar fir and Redwood in USA. Spread of this pathogen has devasting consequences on natural forests, landscape and plant nurseries in USA and other countries. Earlier attempts for characterization based on classical methods such as examining the sexual type, antheridial configuration and sporangium papillation. Using molecular techniques RFLP, RAPD and isozyme analysis, which failed to detect base mutation. Therefore, rapid and accurate microbial identification is essential for any pathogen identification. Kong et al.[19] used PCR-SSCP to identify sequence variation among genetically close relatives of *P.ramourum viz., P.lateralis, P.cactorum, P.citricola, P.heveae, P.nicotiane, P.citrophora* and *P.cinnamom*, SSCP discriminated these pathogens based on their banding patterns using ITS-I primer, and they concluded that SSCP technique can be used as a taxonomic tool for the identification of genetically related microbial pathogens.

Walsh et al.[37] have reported the usage of SSCP, as a technique to set off the differences amongst different medicinally significant opportunistic fungal species and/or genera. The SSCP banding patterns obtained in their study could markedly individualize fungal strains belonging to different genera - *viz., Candida, Aspergilus, Cryptococcus, Rhizopus* etc. Further, the patterns were sufficiently contrast enough even to demarcate between strains of the same genus. The authors also propose the method of SSCP as a cutting edge technique to detect & discriminate between various medicinally important opportunistic fungi, which inturn could play a crucial role in deducing the mechanisms of anti-fungal drug resistance.

Viral identification

Henine et al.[14] have applied the method of long terminal repeat (LTR) based SSCP for the molecular subtyping of HTLV-2 to make up for the less sensitive RFLP analysis that might not distinguish HTLV-2 subtypes comprehensively in HTLV-2 infected populations, whereas a single restriction type was predominant. HTLV-2 is an oncovirus in the *Retroviridae* family, closely related to HTLV-1 type. Though it has not been substantially linked with any disease, instances of, atypical hairy T-cell leukemia and myelopathy-tropical spastic paraparesis like neurolgic diseases (of the HTLV-1),

have had reports of HTLV-2 infection. Seldom is known about the nature and history of HTLV-2 infection and subsequent pathogenicity. In face of this, Henine et al.[14] found the genetic diversity of HTLV-2 worth investigating as it could be used as a tool for viral subtyping in epidemiologic conditions of blood borne, sexual or maternal transmission, and is also handy in screening the occurrence of unknown viral variants by appraising about their Phylogenetic understanding. Their study population included 52 HTLV-2 seropositive blood donors, whereas 32 (16 pairs of male and female) had a long term (≥ 6 months) sexual affiliation. Henine et al.[14] used a sequence (LTR) of 172 bp for the analysis by SSCP. Primary PCR products were retrieved by the amplification of the LTR sequencing using BSQF6 and BSDR3 as primers. Subsequently, a nested PCR amplification of these products was also performed using ^{33}p-end -labeled primers BSSF1 and BSSR2. The SSCP analysis of the nested PCR product was heat denatured and electrophoresed along with a non-heat denatured control sample. The PCR amplified LTR region was also sequenced using pT7Blue vector, and was also comparatively tested by RFLP assay using eight different restriction enzymes. The resulting SSCP analysis data of 52 HTLV-2 samples studied exhibited nine different banding patterns supporting the pre-valence of many different viral genomes in the population. Furthermore, of the 16 sex partner pairs, all 32 HTLV-2 samples had very much similar SSCP resolutions, thereby strongly advocating the presumption that HTLV-2 is transmitted sexually. Still more, the banding patterns of single strand bands in all cases imply that there is one predominantly present viral species in each pattern, thereby backing the presumption of the chief quasispecies lacking in HTLV-2 infected people. Amidst, RFLP analysis could detect the presence of only four restriction types, proving it to be less accurate in comparison with SSCP. Out of these samples, the ones that showed the same restriction type were directed to SSCP evaluation, which in turn could subtype them by patterning into five different types. Also sequencing probing of two dissimilar patterns validated their divergence in terms of the nucleotide changes. Thus, Henine et al.[14] demonstrated a high throughput and adept technique for the sub sterotyping of HTLV-2 stains.

Sampietro et al.[31] have reported an SSCP tool for the characterization of heterogeneity in Hepatitis C virus (HCV) genomes in a test population comprising of 48 HCV infected patients who were on the same kind of maintenance heamodialysis (HD) treatment, and hence forth, they supposed infection to have occurred by nosocomial mode of HCV transmission. HCV is an unknown virus and is the prime cause of Hepatitis in patients introduced to maintenance HD. Diagnosis of HCV was also addressed using reverse transcription followed PCR analysis. But the predominance of a single viral type in a set of population or, of a particular geography, may hinder and limit the utility of this technique. But SSCP method was found to be more inclusive and precise for the same. Serum samples from the patients were tested for anti-HCV-RNA-PCR trial using previously established protocols[2,27], of these PCR products, those that were HCR-RNA positive were denatured and analyzed for SSCP banding patterns. Sequence variation in the SSCP patterns were studied using 5'UTR of HCV and screen for the nucleotide substitution. HCV-RNA results were indicative of 28 cases (58%) to be HCV positive at Enzyme immuno assay (EIA). The SSCP data of these positive samples could resolve and discriminate six different patterns, out of which 3 patterns were more common accounting for 85% of the total observation. They could also validate the patterning seen in SSCP analysis through sequence evaluation and interpret that the most frequent patterns were of, type 4, type 2a and type 1b HCV[16,27,36]. The most frequent being type 4 HCV, rarely found Italians from which the test population was selected. In conclusion, owing to the relative homogeneity of the observed variations in the HD patients treated in the same HD unit, and also the appearance of a rare viral variant in this unit, Sampietro et al.[31] supposed a nosocomial mode of transmission of HCV in the dialytic environment.

Fujioka et al.[8], exercising a combination of reverse transcription-polymerase chain reaction (RT-PCR) and SCCP analyses, have reported a feasible method for the ready classification of different enterovirus genotypes. The primer pair they chose for the amplification was from a highly conserved sequence at the 5´ noncoding region of the enteroviral genomes. SSCP analysis of these amplified products from different serotypes

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of the virus, have been shown to exhibit different electrophoretic profiles. Thereby, stating the handiness of SSCP as a robust tool to effectively diagnose enteroviral infection.

Lin et al.[22] have reported SSCP as a highly dynamic method for the sub typing of human immunodeficiency virus - 1 (HIV-1). Their study reports SSCP to thoroughly resolute and screen between HIV-1 variants even by as little as one nucleotide difference, as reflected in their electrophoretic patterns. Given the seemingly pretentious nature of different HIV-1 strains to be alike (a crucial point to be considered in any immune or drug-based therapy for HIV-1 infection), such a critical appraisal about their characterization, by SSCP, is of prime importance in the course of HIV-1 treatment.

Employing the technique of restriction site polymorphism (RSP) involving a class of 12 restriction endonuceases (REs), Kerr et al.[17], has reported distinct variations amongst human parovirus B19 strains, which supposedly were characterized previously under the same genome type. They also could bridge a linking relationship between two different strains belonging to separate genome types, which meant evidence for the transmission of B19 viral strains, globally. Their results are indicated the usefulness of 4- and 5-bp REs to score the extent of genetic diversity, and it also drives for the need to paraphrase the taxonomy of human B19 viruses by using this series of REs.

SSCP LIMITATIONS AND CONSIDERATIONS

Single–stranded DNA mobilites are dependent on temperature. For best results electrophoresis must be run at constant temperatures.

Sensitivity of SSCP is affected by pH. Double stranded DNA fragments are usually denatured by exposure to basic conditions: a high pH, found that adding glycerol to the polyacrylamide gel lowers the pH of the electrophoresis buffer- more specifically, the tris-borate buffer which increases sensitivity of SSCP[20].

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 even larger fragment size. The presence of glycerol in the gel may also allow a larger DNA fragment at acceptable sensitivity[20].

PCR-SSCP detects the occurrence of base pair mutations in segments of DNA, but does not give any information on types of base changes, which need to be confirmed by sequencing.

ADVANTAGES OF SSCP

SSCP has simplicity in usage without the requirement of special equipment and also the total time needed for PCR-SSCP from extraction of genomic DNA to visualization of the gel is less than 24 h.

Radioactive labeling is not necessary

SSCP is a sensitive, inexpensive and rapid method for detecting sequence variations.

Unlike other techniques such as Denaturing Gradient Gel Electrophoresis (which is specially meant for mutation analysis), SSCP does not have the requirement of GC clamps primers, gradient gel or any other specific and costly apparatus[34].

CONCLUSION

PCR-SSCP analysis is simple compared with other PCR based techniques for mutation detection. In PCR-SSCP mutations are detected by the presence of shifted bands rather than by the absence of signal, as in dot blot hybridization. A few hundred samples may be screened at once, which contrasts to some other techniques in which relatively short sequences are detected-for e.g., restriction enzyme and oligonucleotide probe analyses. The technique has obvious advantages for screening of large numbers of fragments for nucleotide differences compared with a known sequence, and may thus reduce the requirement for costly and laborious nucleotide sequencing. Though the DNA sequencing has become a common technique now-a-days, the equipment is still unavailable in common laboratories and the experiment is still costly, especially when dealing with a large number of samples or stains in molecular biology studies[28,29]. Because of its simplicity and relatively high sensitivity for the detection of sequence variations, SSCP has become one of the most popular mutation detection strategies since it introduction in 1989[28,29,38].

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