Reviews

Applications of polymerase chain reaction-single stranded conformational polymorphism to microbiology

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Introduction

Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) is a simple method for the detection of mutations. The target nucleotide sequence is first amplified by PCR and then separated in single stranded form by electrophoresis in a non-denaturing polyacrylamide gel.¹

The electrophoretic mobility of a DNA molecule in a gel is sensitive to both its size and shape. Under 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 DNA fragment with a mutated sequence shows a change of mobility in polyacrylamide gel electrophoresis when compared with a DNA fragment with the wild type sequence. This occurs as a result of changes in the folded structure (fig 1).

In practice, a few microlitres only of PCR product are mixed with formamide dye and loading buffer, heated to denature double stranded DNA, and loaded on to a vertical polyacrylamide sequencing gel. This is run at a constant 10–20 W for about five hours at usually less than room temperature. The detection of electrophoresed fragments may be performed using various methods; autoradiography requiring prior use of radioactively labelled primers or dNTPs in the PCR,² silver staining,³ detection with biotinylated primers and streptavidin-alkaline phosphatase conjugate,⁴ or ethidium bromide staining.⁵

As mutation detection depends on the conformational changes of the single strand induced by the mutation, its efficiency is sensitive to the physical environment in the gel. For example, the level of cross linking within the gel, the temperature, and the concentration of ions, and solvents. A temperature rise is particularly hazardous for reproducibility, and is avoided by use of a thin gel, air cooling, and use of water jackets on one or both sides of the gel. Separation of single strands is better in polyacrylamide gels with low cross linking, as expressed by %C, the ratio of concentration of N,N'-methylenebisacrylamide to the concentration of total acrylamide monomer.⁶ The

lower this ratio, the softer is the gel, the bigger the pore size, and the greater the sensitivity to conformation. This is probably explained by the increased freedom of polyacrylamide fibres. Gels at 1–2%C and 5–6% total acrylamide monomer are commonly used. The presence of low concentrations of glycerol in a gel may improve separation of mutated sequences. The reason for this is unknown, but glycerol is a weak denaturing agent and this effect may be due to the opening up of the single strands. Glycerol is viscous and reduces mobility, especially at 4°C. However, rarely mutated sequences show mobility shift only in gels without glycerol. In summary, most mutations will be detected in a fragment of less than 200 base pairs by electrophoresis at both room temperature and 4°C, using a gel of 1%C containing 5% total acrylamide monomer, with 5% glycerol.¹

Because of its high resolving power, polyacrylamide gel electrophoresis can distinguish most conformational changes caused by subtle sequence differences. Assuming the method is optimised, sensitivity may be as high as 100% for a fragment ≤ 200 base pairs, but may be as low as 50% for a fragment ≥ 400 base pairs. It may also be possible to predict alterations in single strand DNA conformation caused by nucleotide sequencing, using computer software.⁷

To detect mutations in long segments of DNA, for example 1 kilobase, restriction endonuclease fingerprinting has been used.⁸ This involves PCR amplification of the region of interest, specific cutting with restriction endonuclease, followed by SSCP analysis. This gives not only a restriction map, but also an assessment of the presence of mutations within the resulting fragments. To optimise the method, endonucleases were chosen to give fragments with an average size of 150 base pairs, and 100% of mutations were detected using this method compared with nucleotide sequencing.⁸ Other innovations include multiplex PCR-SSCP, incorporating multiple primer sets,⁹ SSCP analysis of RNA¹⁰ and use of fluorescence PCR primers followed by analysis with an automated DNA sequencer.¹¹

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Figure 1 Diagram showing the principle of single stranded conformational polymorphism (SSCP) analysis.

Applications of PCR-SSCP to microbiology

PCR-SSCP analysis has been used in studies of various different micro-organisms (table 1).

MICROBIAL IDENTIFICATION

Bacterial identification

A group in Holland amplified a variable region of the 16S rRNA sequence from bacterial cell

Table 1 Microbiological applications of PCR-SSCP

Microbial identification

Bacterial 16S ribosomal DNA¹²
Fungal 18S ribosomal DNA¹³
Enteroviruses¹⁴

Molecular epidemiology

Campylobacter jejuni (see text)
Staphylococci¹⁵
*Mycobacterium leprae*¹⁶
Parvovirus B19^{3, 18}
Human papillomavirus type 16^{20, 21}
Epstein-Barr virus^{22, 23}
Hepatitis C virus^{24, 26}
Mumps virus²⁷
Human T cell lymphotropic virus type 2²⁸
Human immunodeficiency virus type 1²⁹

Prediction of microbial susceptibility to chemotherapeutic drugs

*Mycobacterium tuberculosis*³⁰⁻³²
*Staphylococcus aureus*³³
*Bacillus subtilis*³⁴
Hepatitis C virus^{25, 35}

lysates using primers complementary to conserved sequences. PCR products were then analysed using SSCP and detected using silver staining. Over 100 strains, spanning 15 genera and 40 species, were examined with two primer sets, P11P-P13P and ER10-ER11. Genus and species level identifications were obtained. For example, species specific patterns were obtained for *Clostridium* spp, *Listeria* spp, *Pseudomonas* spp, and *Enterobacter* spp.¹²

Fungal identification

Walsh *et al*¹³ amplified a 197 base pair region from the 18S rRNA gene of various fungi, which is common to all medically important fungi. SSCP analysis showed identical banding patterns for *Candida albicans*, *C tropicalis*, and *C parapsilosis*, and all strains within each species showed the same pattern. However, these patterns differed considerably from those of the genus, *Aspergillus*. Strains of *A fumigatus* and *A flavus* were consistently differentiated. SSCP patterns for *Cryptococcus neoformans*, *Pseudallescheria boydii*, and *Rhizopus arrhizus* were sufficiently unique to permit distinction from those of *C albicans* and *A fumigatus*.

Figure 2 SSCP analysis of PCR products showing five distinct B19 strain types with respect to the region amplified (nt 1399–1682).³

Viral identification

Enteroviral genotypes were identified in 14 serotypes of enteroviruses including Coxsackie A and B viruses, echoviruses, and polioviruses by reverse transcriptase (RT)-PCR amplification of a 154 base pair highly conserved non-coding sequence at the 5' end of the enteroviral genome. These fragments were analysed by SSCP, giving discriminatory patterns which permitted rapid enteroviral identification.¹⁴

MOLECULAR EPIDEMIOLOGY

Campylobacter jejuni

To investigate the molecular epidemiology of prolonged, severe *C. jejuni* infection in a man with hypogammaglobulinaemia, four blood culture and two faecal strains, isolated over a 16 month period, were analysed. Isolates were characterised at the subspecies level by Penner serology, Preston biotyping, Preston phage typing, *HaeIII*-digested total genomic DNA detected with both E3CJC2 and 16S ribosomal DNA probes, and analysis of a PCR amplified, 1.7 kilobase pair region of the *C. jejuni* *flaA* gene by *DdeI* digestion alone and with SSCP analysis detected by silver staining. Using these methods, at least two different infecting strains were demonstrated; one strain that persisted throughout the 16 month period and another transient strain, suggesting reinfection from a different source. SSCP analysis was the most discriminatory of the methods, showing an altered form of the persistent strain with subtle modifications of the hypervariable region of the *flaA* gene in one isolate (Moore JE *et al.* Abstract presented at the 95th general meeting of the American Society for Microbiology, Washington, DC: 21–25 May 1995).

Staphylococci

Molecular typing of the methicillin resistance determinant (*mec*) based on the length of the *mec* associated hypervariable region was performed on 61 isolates of methicillin resistant *Staphylococcus aureus* (MRSA), 15 methicillin resistant *S. epidermidis*, and 11 methicillin resistant *S. haemolyticus* using PCR. Five, three, and two genotypes were seen within each of these groups, respectively, based on the size of the PCR product. PCR products from 14 of 15 methicillin resistant *S. epidermidis* were the same size as those from MRSA isolates, and

their sequence was identical, as confirmed by SSCP and sequencing. In methicillin susceptible isolates the *mec* gene was not amplified. This method may be valuable for investigation of nosocomial outbreaks of infection.¹⁵

Mycobacterium leprae

The 282 base pair ribosomal intergenic spacer region of 75 *M. leprae* isolates from patients with leprosy, healthy subjects, armadillos, and mouse footpads was shown to be 100% conserved by PCR-SSCP and nucleotide sequencing.¹⁶

Parvovirus B19

A 284 base pair region within the NS1 gene of 50 strains of human parvovirus B19, the cause of erythema infectiosum, was analysed using PCR-SSCP. SSCP detected all mutations. Five SSCP types were found and confirmed by nucleotide sequencing (fig 2). Two SSCP types accounted for 92% of strains. Although no correlation was seen between genotype and clinical manifestations, there was a correlation between genotype and both geographical location and year of isolation.³ This is consistent with global spread and replacement of B19 virus strains.¹⁷

The NS1 gene of B19 strains isolated from seven patients with persistent B19 infection (cases 24, 39, 40, 41, 46, 50, 51) have also been analysed using PCR-SSCP.¹⁸ In two of the four cases for which both acute and follow up PCR product was available, the SSCP type of the follow up product was different from that of the acute product (cases 40, 41) (fig 3). The SSCP type of follow up products for cases 24, 39, 40, 41, 50 was also similar. Two B19 virus types with respect to the region studied were shown in one patient with arthralgia and chronic fatigue syndrome at follow up assessment (case 51) (fig 3). Therefore, this method showed nucleotide change occurring during persistent B19 infection, during which a virus clone with an advantage for persistent growth might have become predominant. Nucleotide sequence analysis of Aleutian mink disease parvovirus indicated that this virus has a considerable degree of variability and that several virus types may be present simultaneously in infected animals.¹⁹

Human papillomavirus type 16

HPV causes cervical cancer and anal warts, with types 16 and 18 being by far the most carcinogenic types. Analysis of the non-coding region of the HPV16 genome by PCR-SSCP showed that in two pairs of monogamous homosexual men with anal warts, the same virus type occurs in both partners, and that this type differed from that isolated from other pairs of partners.²⁰ The virus was shown to establish a persistent infection in which a single variant predominates, and that coinfection with additional variants does occur but represents a transient phenomenon.²¹

Epstein-Barr virus (EBV)

Epstein-Barr virus (EBV), the cause of infectious mononucleosis, exists as two wild type

Figure 3 SSCP analysis of PCR products from the serum of seven patients with persistent B19 infection. Each specimen showed double stranded DNA of 284 base pairs (nt 1399–1682) on conventional agarose gel electrophoresis.¹⁸

families based on diversity of the EBNA-2 gene. Type A immortalises B cells more efficiently in vitro and infects immunocompetent persons more frequently than type B, which more frequently infects immunocompromised subjects and is associated with lymphoid neoplasms. Based on PCR amplification of a 190 base pair fragment spanning the sites of six specific point mutations which determine strain type, SSCP assay grouped strains as either A or B.²²

The post-transplant lymphoproliferative disorders (PT-LPD) are a heterogeneous group of B cell neoplasms that arise in the setting of immunosuppressive treatment, and are associated with EBV infection. PCR-SSCP analysis of isolates from cases of PT-LPD showed, surprisingly, that in 24 (89%) of 27 cases these strains were characteristic of type A, and that none of the lesions examined harboured EBV type B.²³

Hepatitis C virus

Hepatitis C virus (HCV) populations in vivo exist as a mixture of heterogeneous viruses called quasispecies which vary in the so-called hypervariable region (HVR) of the envelope 2 gene, a region known to contain antibody epitopes. HCV quasispecies populations change during the natural course of chronic infection. In one study serial serum samples were obtained from nine patients with chronic HCV infection²⁴; RT-PCR-SSCP was used to analyse the envelope gene. This showed that within each patient the HCV population consisted of one to six quasispecies, which changed sequentially in eight of nine patients; both gradual selections and complete replacements were observed. Phylogenetic analysis revealed continuous accumulation of mutations during chronic HCV infection. Another study using RT-PCR-SSCP has shown that the diversity of HCV quasispecies becomes more complex as the disease stage progresses, and that chronic active hepatitis with more complex viral diversity shows reduced interferon responsiveness.²⁵

Although blood transfusion is the main route of HCV transmission, nosocomial transmission within an Italian dialysis unit was demonstrated using RT-PCR-SSCP analysis of the 5' untranslated region of the HCV genome in 28 isolates from haemodialysis patients compared with 25 epidemiologically unrelated isolates from patients with liver disease²⁶; six SSCP patterns were demonstrated among isolates

from dialysis patients compared with 16 possible SSCP patterns among the control patients with liver disease. Subsequent sequence analysis showed that the most common HCV type among the dialysis patients was type 4, which is rare in Italy.

Mumps virus

The phospho-protein gene of four different mumps vaccine strains (Urabe, Torii, Hoshino, Miyahara), 12 wild type mumps strains and a laboratory strain (Enders) were shown to be distinct by PCR-SSCP. Viruses isolated from patients who developed aseptic meningitis four to six weeks after measles-mumps-rubella (MMR) vaccination showed identical SSCP and sequence patterns with the particular vaccine strain used for immunisation.²⁷

Human T cell lymphotropic virus (HTLV) type 2 PCR-SSCP was used to analyse a highly variable region in the long terminal repeat of 52 HTLV-2 strains. Identical SSCP patterns were obtained from each of 16 couples, suggesting the presence of similar viral genotypes and therefore supporting the likelihood of sexual transmission of HTLV-2 in each of these pairs. SSCP was much more discriminatory than restriction fragment length polymorphism (RFLP) analysis, the currently used method for HTLV-2 typing.²⁸

HIV type 1

To characterise HIV-1, SSCP analysis was applied to two hypervariable regions of HIV-1 nucleic acids in plasma and peripheral blood mononuclear cells (PBMC) from 16 patients and to 15 PBMC co-cultures and six plasma cultures prepared from these specimens. The HIV-1 env target region produced in culture diverged from that in the inoculum in 18 of 21 instances.²⁹ The technique emphasised the quasispecies nature of HIV-1 infection, a phenomenon which may account for the existence of HIV-1 clones with functional diversity and the emergence of drug resistant variants.

PREDICTION OF MICROBIAL SUSCEPTIBILITY TO CHEMOTHERAPEUTIC DRUGS

Mycobacterium tuberculosis

A major hindrance to the success of global control of tuberculosis, with its recent development of antibiotic resistance, has been the time taken to culture the organism. However, with nucleic acid amplification, detection may now be rapid. Once the presence of the organism

has been confirmed, PCR-SSCP has been used to analyse mycobacterial genes whose products are the target for specific antibiotics, and in which mutations reduce the antibiotic susceptibility of the organism. Rifampicin inhibits bacterial RNA polymerase, the β subunit of which is encoded by the *rpoB* gene. A number of mutations causing rifampicin resistance are localised to the *Rif^r* region and were detected by PCR-SSCP with sensitivities of 100%³⁰ and 67%³¹ detection of mutations correlated with resistance as detected by conventional methods.

Isoniazid has a bactericidal action against mycobacteria, but its precise mode of action has long been unknown. Recently, Heym *et al.*³² used PCR-SSCP to examine the *katG* gene, encoding the haem containing enzyme catalase-peroxidase, of isoniazid resistant *M. tuberculosis*, and discovered missense mutations and gene deletions resulting in abnormal catalase-peroxidase enzymes. As prompt, appropriate treatment will be crucial in global control of tuberculosis, these rapid sensitivity testing methods are an important advance.

Staphylococcus aureus

Fluoroquinolones act by inhibiting bacterial DNA gyrase, an enzyme which is responsible for DNA supercoiling; fluoroquinolone resistance may be mediated by mutation in the genes encoding the DNA gyrase. Thirty six clinical isolates of *Staphylococcus aureus* (29 fluoroquinolone resistant and seven fluoroquinolone susceptible) were examined using PCR-SSCP for point mutations of the *gyrA* gene. Fluoroquinolone resistance was closely associated with six types of mutation in the *gyrA* gene. All mutations were detected within three hours of PCR amplification.³³

Bacillus subtilis

Munakata *et al.*³⁴ examined a 163 base pair fragment of the 5' portion of the *gyrA* gene in 607 nalidixic acid resistant strains of *Bacillus subtilis* using PCR-SSCP and sequencing. SSCP patterns of resistant strains differed from those of susceptible strains. Thirteen *gyrA* alleles were identified; eight were single base pair substitutions, four were substitutions of two consecutive base pairs, and one was a substitution of three consecutive base pairs.

Hepatitis C virus

It has already been mentioned that SSCP demonstrated that HCV associated chronic active hepatitis with wide viral diversity shows reduced interferon responsiveness.²⁵ Enomoto *et al.*³⁵ investigated the fluctuation of HCV quasiespecies during interferon treatment using SSCP. In 13 of 16 patients whose infection was refractive to interferon, the predominant HCV population was replaced with other quasiespecies during treatment. In nine patients one quasiespecies which had existed before the treatment with interferon, became predominant after the treatment. It was concluded that sensitivity to interferon differs among HCV quasiespecies and that interferon selects resist-

ant HCV strains. Existence of such quasiespecies was associated with treatment failure.

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 hybridisation. A few hundred bases may be screened at once, which contrasts to some other techniques in which relatively short sequences are detected—for example, 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. PCR-SSCP is a relatively new technique, but has already made a substantial contribution to microbiology.

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