 Efficiency and cost effectiveness: PAGE-SSCP versus MDE and Phast gels for the identification of unknown β thalassaemia mutations

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Background: Prenatal diagnosis for β thalassaemia has proved to be very effective in preventing the birth of an affected child and hence in controlling the disease. The success of prenatal diagnosis depends on the delineation of the underlying mutations in the population at risk. Each population carries a limited number of frequent defects (89–91%) and a variable number of rare alleles (4–5%), whereas 2–3% of alleles remain uncharacterised. To offer prenatal diagnosis when the parental mutation is unknown, the application of a non-specific detection method (such as single stranded conformational polymorphism (SSCP)) to localise the mutation, followed by direct sequencing of the amplified gene sequence, is required. With this objective in mind, this study was designed to devise the best protocol and system of SSCP for the rapid screening of unknown mutations in the β globin gene.

Methods: To detect mutations in this disease, three different systems—Phast gels, MDE gels, and polyacrylamide gels—were used under varying conditions.

Results: Polyacrylamide gels were found to be the most efficient, both in terms of resolution and cost. Polyacrylamide gels are the most rapid, efficient, reliable, and cost effective means for DNA mutation analysis of the β globin gene.

Materials and methods

Five primer sets encompassing the entire β globin gene were used for SSCP analysis (table 1). Figure 1 shows the primer locations and orientations. PCRs were carried out in a Perkin Elmer thermocycler 480. A 100 ng aliquot of genomic DNA was amplified using 15 pmol of each primer, 2.5 μmol of MgCl₂ (Bangalore Genei, India), 10 mmol of dNTPs (Bangalore Genei), and 1 Uof Taq polymerase (Bangalore Genei). This sample mix was subjected to a hot start, consisting of denaturation at 95°C for three minutes, first annealing at 65°C for one minute, and extension at 72°C for 1.5 minutes, followed by 24 cycles of 93°C for one minute, annealing at 65°C for one minute, and extension at 72°C for 1.5 minutes. The results were visualised using a 1.5% agarose gel and ethidium bromide staining.

The band of interest was cut out, 600 μl of 1% silica in 6M KI was added, and the tube was centrifuged at 10 000×g for five minutes. The supernatant was discarded and 700 μl of 50% ethanol was added, after which the tube was vortexed vigorously and centrifuged at 10 000×g for five minutes. The supernatant was again discarded and 700 μl of acetone was added, the sample mixed, and centrifuged again. The supernatant was aspirated, the pellet was air dried, and 20 μl of water was added. The sample was then mixed, centrifuged at 10 000×g, and the supernatant (DNA) collected.

SSCP conditions: Phast system

SSCP analysis was performed with the Phast system on homogeneous polyacrylamide mini gels type 20 (20% polyacrylamide) using native buffer strips (Pharmacia Biotech). The radioactively labelled PCR product was mixed with SSCP loading dye at a ratio of 1:1. The samples were denatured at 94°C and snap cooled on ice. The Phast gel was pre-run at 100 V, 10 mA, 2.5 W, and 7°C for 100 Vh. Samples (0.5 μl) were loaded at 25 V, 1 mA, 2.5 W, and 7°C for 5 Vh and electrophoresis was carried out at 400 V, 10 mA, 2.5 W, and 7°C for 400 Vh. The gel was exposed to x ray film and developed.

MDE gels

Large (sequencing format) 0.5× MDE gels were prepared with 0.6x TBE. Samples were diluted (1/5) with formamide dye (Biorad), denatured at 95°C for two minutes, and quenched on ice. The denatured samples (3 μl aliquots) were loaded and

ORIGINAL ARTICLE

β thalassaemia is the most prevalent monogenic disease in India. The detection of unknown β globin gene mutations requires a comprehensive scanning method. Single stranded conformational polymorphism (SSCP) is commonly used because of its technical sensitivity and feasibility. SSCP has been reported to be the most sensitive test when the DNA fragment size is 150–300 bp in length. It is recommended that large format gels (5–50 cm) are used, that the gels have a low pH and a high percentage of acrylamide, and that the electrophoretic buffer is of high ionic strength and low pH. The minimum of polymerase chain reaction (PCR) cycles should be carried out and the high dilution of the resultant product should also be taken in account. Gels should be run either at room temperature or at 4°C, with or without glycerol.

“The detection of unknown β-globin gene mutations requires a comprehensive scanning method”

The various SSCP systems that have been used are large native polyacrylamide gels, vinyl polymer, hydrolink-MDE™ (FMG Bioproducts, Rockland, Maine, USA), and the Phast system™ (Pharmacia Biotech, Uppsala, Sweden). We have compared and assessed the efficacy and cost effectiveness of the above SSCP methods for the detection of mutations using a panel of known β thalassaemia mutations.

Abbreviations: PCR, polymerase chain reaction; SSCP, single stranded conformational polymorphism
electrophoresis was carried out for 14 hours at constant power (8 W), at room temperature (in an air conditioned room at 20–25°C).

Polyacrylamide gels
A 10% polyacrylamide gel was prepared and pre-run for one hour at a constant power of 60 W. A 1µl aliquot of the α32dCTP labelled PCR products was added to 9 µl of loading dye containing 96.5% formamide. Samples were denatured at 95°C for five minutes and chilled on ice. These denatured samples (3 µl aliquots) were loaded on to the gel and run at room temperature in 1× TBE for 16 hours, at 8 W. An additional 1.5% of formamide was used to improve the resolution.

RESULTS AND DISCUSSION
Mutational analysis of the β globin gene using the Phast and MDE gels produced mutant bands were as intense as the normal ones and the separation was good (figs 2, 3). The Phast system was very rapid—the entire procedure was completed in less than two hours.

“Using a higher concentration of formamide (96.5%) than normal (95%) gave better results, with no smiling bands”

Different conditions were tested with the polyacrylamide gels, such as pre-running the gels, using denaturants, adding glycerol, and varying the ionic strength of the electrophoretic buffer and the percentage of the polyacrylamide. The

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<th>Table 1</th>
<th>The sequence and size of the primers used for single stranded conformational polymorphism analysis of the β globin gene</th>
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<td>Fragment</td>
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Figure 1 A schematic representation of the human β globin gene with the location of the primers, and the region and the size (bp) of the fragments they amplify. IVS, intervening sequence (intron).

Figure 2 Autoradiograph of the Phast gel. Lanes 1 and 6, samples heterozygous for the CD 41/42 β thalassaemia mutation; lanes 2 and 3, and 5, samples homozygous for the CD 41/42 β thalassaemia mutation; lane 4, normal.

Figure 3 Autoradiograph of the MDE gel. Lanes 1 and 2, normal; lanes 3 and 4, samples heterozygous for the CD 41/42 β thalassaemia mutation.

The polyacrylamide and Hydrolink-MDE gels were transferred to Whatman 1MM and 3MM paper, respectively. They were then dried in a gel drier (Rapid Dry, Atto, Japan), exposed to x ray film (Kodak) at ~70°C for 24 hours, and developed.

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resolution was good when the gel was pre-run at 60 W for one hour. Using a higher concentration of formamide (96.5%) than normal (95%) gave better results, with no “smiling” bands. The band resolution was not affected by the addition or absence of 5% glycerol. Three ionic strengths of the TBE buffer were used: 0.5×, 1.0×, and 1.5×. Normal strength (1×) TBE yielded the sharpest SSCP bands using 8–12% gels. The sharpest and most consistent bands were obtained with 10% gels (fig 4).

Thus, polyacrylamide gels were found to be the most rapid, efficient, reliable, and cost effective method for DNA mutation analysis in β thalassaemia.

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REFERENCES

ECHO

Protein kinase regulates IL16 transcription in arthritis

Molecular researchers have suggested that pathways dependent on protein kinase C may regulate transcription of interleukin 16 (IL16), a proinflammatory cytokine abundant in arthritic joints. They compared the effect of various chemical agents on steady state (IL16) mRNA transcripts in growing synovial fibroblasts from six patients with rheumatoid arthritis (RA) and three with osteoarthritis (OA). The reverse transcriptase PCR method they used enabled them to obtain results that were semiquantitative.

Early passage synovial fibroblasts from patients with RA or OA transcribed IL16 mRNA when incubated with growing medium without additives. Protein kinase inhibitor staurosporine enhanced IL16 steady state mRNA in both types of synovial fibroblasts and specific protein kinase C activator phorbol-12-myristate-13-acetate reduced transcription. Other agents—the calcium ionophore ionomycin, protein kinase A stimulator cyclic AMP, and G protein activator MAS-7—gave minor, variable responses. Phosphatase inhibitor okadaic acid and protein kinase inhibitor H-7 dihydrochloride reduced mRNA transcripts, maybe because of their killing the fibroblasts. This response pattern suggests that IL16 is regulated by protein kinase C dependent mechanisms, say the researchers.