

INTRODUCTION

The development of PCR for amplifying DNA sequences has raised the possibility that VNTRs and other DNA polymorphisms might be analyzed using this approach. Some methods for detecting single base changes in DNA after PCR amplification have been proposed as ribonuclease cleavage, denaturing gradient gel electrophoresis and the PCR amplification of specific alleles. These methods have rarely been used in practice because they involved complicated methodology or else, its use in forensic or clinical casework has not been proved until now. Here we propose a method for detecting DNA polymorphisms, not only in coding DNA but also in repetitive DNA (e.g. minisatellites). The method involves a DNA extraction with a chelating resin, PCR amplification, SDS-PAGE in gradient non-denaturing gels and silver staining. Its usefulness is exemplified by typing the HLA-DQA1 system and the VNTR in the position D1S80.

MATERIAL AND METHODS

DNA was extracted from 4 μ l blood by a method described previously (Singer-Sam et al. 1989) using a chelating resin (Sigma, St Louis, MO). The total extraction time was 45 min. The oligonucleotides used as primers for HLA-DQA1 amplification were GH26 (5' GTGCTGCAGGTGAAACTTGTACCAG 3') and GH27 (5' CACGGATCCGGTAGCAGCGGTAGAGTTG 3') and for pMCT118 were 5' GAAATGGCCCTCCAAACACTGCCCGCCG 3' and 5' GTCTTGTGGAGATGCACGTG-CCCCCTTGC 3'. These primers amplified a 16-base pair repeating unit, the different alleles containing 15-36 repeating units. Each sample amplified contained 1-10 ng DNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 2.5 units of AmpliTaq DNA Polymerase (Cetus), 0.188 mM of each dNTP and 0.2 μ M of each primer. The volume of each sample was 50 μ l. The cycling reaction was done in a programmable heat block (Perkin-Elmer/Cetus) set to heat at 94°C for 1 min (denature), cool at 60°C for 30 sec (anneal), and incubate at 72°C for 30 sec (extend). After 32 repetitions, the samples were incubated an additional 7 min at 72°C. PCR amplification of pMCT118 was achieved by the method described by Budowle et al (1990) with slight modifications. Each amplified sample contained 1-10 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 2.5 units of AmpliTaq DNA Polymerase, 1 μ M of each primer and 200 μ M of each dNTP, for a total volume of 50 μ l. The PCR was carried out for 30 cycles. Each cycle was one minute at 95°C for denaturation, one minute at 65°C for primer annealing, and eight minutes at 70°C for primer extension. Electrophoresis was carried out in PhastGels Gradient 10-15 and 8-25 (Pharmacia-LKB) using the PhastSystem. Polyacrylamide gels (5 x 5 cm) have a 13 mm stacking gel zone, at a gel concentration of 4.5% and cross-linking of 3%, and a 32 mm separation gel zone with a continuous 10-15% or 8-25% gradient with 2% cross-linking. The buffer system in the gels is of 0.112 M acetate and 0.112 M Tris, pH 6.4. The electrode buffer contained 0.20 M tricine, 0.20 M Tris and 0.55% SDS, pH 7.5. The buffer strips are made of 2% agarose (Agarose IEF, Pharmacia-LKB). Samples (1 μ l of amplified product) were applied to the cathodic end of the gel. Electrophoresis was carried out at 50 V/cm, 10.0 mA and 3.0 W for a total of 50 volt-hours. Detection of the PCR amplified products was made with the silver staining method of Heukeshoven and Dernick (1988). Alternatively, normal size gels (10x20x0.4 cm) with the same discontinuous buffer system were used. A 123 bp DNA ladder (Gibco-BRL) was used as size marker. For comparison dot-blot analysis with HLA-DQ α AmplyType (Cetus) was carried out using protocols provided by the manufacturer.

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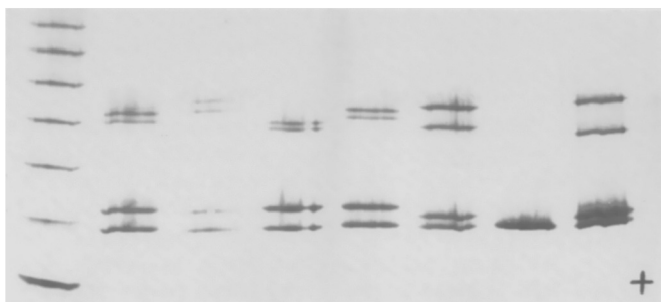


Fig.1. SDS-PAGE of PCR amplification of HLA-DQA1 locus. From left to right HLA-DQA1 genotypes 3-4; 4-4; 2-3; 1.1-4; 1.2-4; 1.1-2; 1.2-4 and 123bp ladder marker. Electrophoresis was carried out in PhastGel 10-15 for 100Vh at 15 C

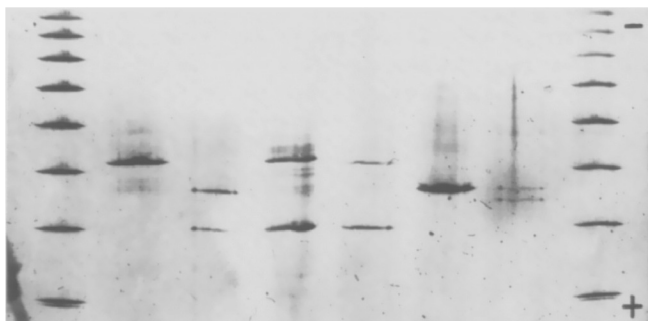


Fig. 2. SDS-PAGE of PCR amplification of locus D1S80. Electrophoresis was carried out in PhastGel 10-15 for 100Vh at 15 C. Samples are 2µl 5 years old bloodstains

RESULTS AND DISCUSSION

Limitations of PCR dot-blot method for HLA-DQA1 typing are, first, that commercially available systems are expensive since they involved immobilized ASO probes and, second, the number of ASO probes do not permit the identification the entire variation of the system and only the more common alleles. The electrophoretic method reported overcomes such limitations, since single substitutions can be detected and enzymes or probes are not necessary. A complete correlation between the electrophoretic patterns of HLA-DQA1 phenotypes and dot-blot results was observed. HLADQA1 products differing only 4 bp substitutions such as the alleles DQA1*0201 and DQA1*0301 can be distinguished in homoduplexes and DQA1 alleles differing a single base substitution in heteroduplexes such as the subtypes DQA1*0101 and DQA1*0103 which can be distinguished in any heterozygotes with other DQA1 alleles (Fig.1). With reference to the electrophoretic method, clear separations were obtained only when discontinuous electrophoretic systems were used. The use of 0.112M acetate (leading ion) and 0.112M Tris, pH 6.4 as buffer system in the gels, and 0.20M tricine (trailing ion), 0.20M Tris and 0.55% SDS, pH 7.5 as buffer in the agarose strips (bridge) gave particularly good results. Homogeneous polyacrylamide gels provided poor resolutions. Only gradient polyacrylamide with stacking gels can be used to obtain a good result. Optimal resolution are obtained with the gradient T=10-15, with a stacking

gel of T=4.5%. Tas (1990) recently proved that DNA molecules are separated readily and rapidly by gel electrophoresis with SDS in a conventional static electric field. We have here observed the same effect for small DNA molecules, HLA-DQA1 cannot be typed if SDS is not added at least in the agarose bridge buffer. Furthermore these small molecules migrated during SDS-PAGE faster than proteins of identical molecular mass, and thus, the number of volthours habitually used for conventional SDS-PAGE separation of proteins has to be considerably shortened. With SDS, DNA molecules form sharper bands and separate better, and this effect can be used for analyzing other DNA polymorphisms. The use of automatized systems for miniaturized gels such as the PhastSystem are particularly recommended, firstly because polyacrylamide gel miniaturization improves protein visualization and increases the sensitivity for detecting low abundance components, and secondly because all variables are controlled, and so results can be always reproduced and samples do not have manipulated by adding bromophenol blue since migration can be controlled by counting the number of volthours. An additional advantage of using the PhastSystem is the possibility of controlling the temperature. Temperature control is essential since the electrophoretic behaviour of heteroduplexes critically depends on it and reproducible results are obtained only when bands are separated at the same temperature. An optimal resolution is obtained between 3 and 15°C. In any case reproducible and short separations can be carried out with the proposed methodology, even when the method is applied to forensic casework. Important advantages of the proposed method for typing the HLA-DQA1 system over commercially available dot-blot systems with ASO probes are the time needed for the entire process (5h for 10h), the cost (at least ten times less) and the sensitivity (positive results were obtained with low amounts of amplified products given dot-blot negative results). The system can also be applied to the analysis of VNTRs and so pMCT 118 alleles can be easily recognized. PhastGel 10-15 and the same electrophoretic discontinuous buffer system gives the best results (Fig.2). Single tandem repeats (16 bp) can be distinguished and the method can used in forensic casework with the advantage over previously published methods, that results can be obtained in only 5 h including extraction. Heteroduplexes are not observed after pMCT118 amplification and the temperature control is not essential in this case. The factors influencing the generation of recombinants are not known and our system can be used as a model to study this fact and the electrophoretic behaviour of small DNA molecules.

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