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HLA-DQA1 allele typing by nonisotopic PCR-LIS-SSCP

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Abstract

In the present study we used a simple and reliable method for HLA-DQA1 allele typing based on the single-stranded conformation polymorphism (SSCP) properties of DNA molecules obtained by PCR. The technique consists of PCR amplification of a DNA fragment comprising the second exon of the HLA-DQA1 gene, amplicon denaturation using a low ionic strength solution (LIS), and electrophoresis on a small native polyacrylamide gel, followed by a rapid silver staining procedure. In order to validate the technique and to obtain the allele patterns for the DQA1 gene, 50 cervical samples were typed using this methodology and the commercial Amplitype® HLA DQA1 Amplification and Typing kit. All the alleles detected with the kit were characterized by the LIS-SSCP approach. This procedure proved to be useful for population screening and typing of the DQA1 gene as well as for detecting new alleles or mutations in the donor-recipient molecular matching of HLA class II genes.

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The human leukocyte antigen (HLA)-DQ molecule is a glycoprotein heterodimer composed of one α-chain (encoded by the HLA-DQA locus) and one ß-chain (encoded by the HLA-DQB locus). These antigens are expressed on the surface of antigen-presenting cells and play a key role in the immune recognition of foreign as well as self-produced antigens (1). The polymorphisms defining the alleles of this HLA class II gene are located in a 242-bp region (or 239 bp for alleles 2 and 4) within the second exon of the HLA-DQA gene. Eight alleles have been identified and are designated as DQA1*0101, *0102, *0103, *0201, *0301, *0501, *0401, and *0601 (2).

HLA class II DNA typing has become very important in the fields of basic and clinical medicine, especially in HLA disease association and donor-recipient matching for transplantations. Also, HLA-DQA1 is the most extensively characterized PCR-based system using the reverse dot-blot format for the analysis of genetic identity in forensic medicine (3).

As described by others (4), LIS-SSCP is referred to as the low ionic strength single-stranded conformation polymorphism procedure, and is based on the diversity in the electrophoretic mobility of ssDNA formed by heat denaturation in low ionic strength solutions. This method has been used to detect DNA polymorphisms, including point mutations at a variety of positions in DNA fragments belonging to the HLA-DRB1, -DQB1, and -DPB1 genes (4).

The classical SSCP methodology, although sensitive, is both time consuming and cumbersome. There are several reports describing nonisotopic protocols for SSCP analysis (5,6), but all of these methods require expensive reagents or equipment such as small-format PhastGel, the PhastSystem instrument and Hydrolink-MDE, or the use of composite agarose gels (7). In the present study, we propose a method for HLA-DQA1 allele typing by nonisotopic SSCP analysis on standard silver-stained polyacrylamide minigels electrophoresed at low temperature (4ºC in a conventional refrigerator). In order to determine the patterns for each allele, the samples were typed for DQA1 in parallel using the LIS-SSCP protocol and the Amplitype® HLA DQA1 Amplification and Typing kit (Perkin-Elmer, Branchburg, NJ, USA).

Genomic DNA was obtained from a total of 50 cervical samples. Samples were centrifuged at 3000 rpm for 10 min, and suspended and washed twice with 1 ml PBS. The pellets were digested in 300 µl of digestion buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1% Triton X-100 and 0.5% Tween 20) containing 200 µg/ml proteinase K (Promega, Madison, WI, USA), for 3 h at 56ºC and boiled for 10 min for proteinase inactivation. The samples were quantified and the DNA concentration was adjusted to 5 ng/µl. All samples were stored at -20ºC until the time for use.

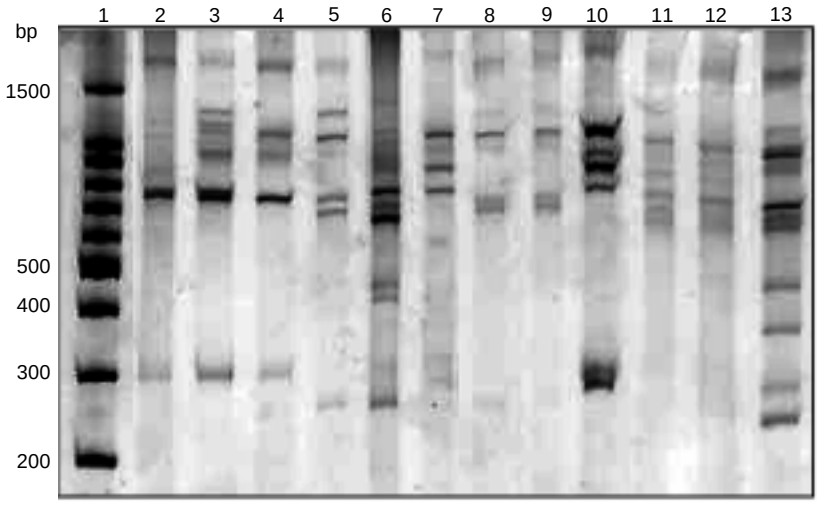

Figure 1. PCR-LIS-SSCP analysis of HLA-DQA1 group alleles. *Lane 1*: Molecular weight marker (100-bp DNA ladder; Promega); *lane 2*: DQA1*0101+0103; *lane 3*: DQA1*0102+0103; *lane 4*: DQA1*0102+0102; *lane 5*: DQA1*0101+0501; *lane 6*: 0101+0201; *lane 7*: DQA1*0102+0301; *lane 8*: DQA1*0501+0501; *lane 9*: DQA1*0501+0501; *lane 10*: DQA1*0102+0301; *lane 11*: DQA1*0301+0501; *lane 12*: DQA1*0301+0501; *lane 13*: DQA1*0103+0501. Electrophoresis was carried out on a 10% gel at 4ºC for 5 h.

PCR amplification was carried out in a final volume of 50 µl using 1.25 units of *Taq* DNA polymerase (Promega), 2 mM $MgCl_2$, 200 µM of each dNTP in PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), and 25 pmol of the following primers: *GH26* 5'-GTGCTGCAGGTGTAAACTTGTACCAG-3' and *GH27* 5'-CACGGATCCGGTAGCA GCGGTAGAGTTG-3' as previously described (8). The amplicon covers the second exon of the HLA-DQA gene (242 bp) and the primers are the same as those used in the commercial kit.

The reactions were cycled as follows: 1 cycle of 94ºC for 2 min, 32 cycles of 30 s at 94ºC, 30 s at 63ºC, 30 s at 72ºC, and finally 1 cycle of 72ºC for 5 min for chain elongation. The amplified fragments were detected by electrophoresis on a 2% agarose gel stained with SYBR-Green I® (Molecular Probes, Eugene, OR, USA).

Four microliters of the PCR product was added to 10 µl of LIS solution (10% sucrose, 0.01% bromophenol blue, and 0.01% xylene cyanol FF) corresponding to 14 µl for gel loading. The mixture was incubated for 10 min at 97ºC and applied to a 10% polyacrylamide minigel (acrylamide/bisacrylamide 39:1). The electrophoresis apparatus was maintained in a standard refrigerator at a constant temperature of 4ºC. Electrophoresis was carried out using 1X TBE (45 mM Tris-borate/1 mM EDTA) at 45 mA for 5 h. SSCP patterns appearing on the gel were detected by a rapid silver staining technique (9).

The reference DNAs were obtained by typing the samples with the commercial kit and searching for the homozygotes for each

particular allele. The concordance between our typing results and the commercial kit were 100%, and a total of 8 DQA1 alleles in homozygous and heterozygous combinations were all correctly identified (Figure 1). The methodology presented here showed no reduction in sensitivity when compared with the commercial kit. In addition, the LIS-SSCP protocol permits the detection of unknown alleles or point mutations, a fact not feasible when using the dot-blot or reverse dot-blot format. The investigated samples were obtained from both fresh and fixed tissues, showing a remarkable sensitivity and specificity regardless of the quality and source of the obtained DNA.

This simple procedure could be immediately applicable, since the equipment required is available in many laboratories. The use of a standard refrigerator avoids the need for expensive electrophoresis devices with liquid circulation for temperature control. Moreover, the PCR-LIS-SSCP technique does not require the large number of synthetic oligonucleotides necessary for other methodologies such as the PCR-SSP and PCR-SSO techniques and is thus both time and cost effective.

This method has also been successfully applied to human papillomavirus typing in a group of samples investigated in other studies conducted in our laboratory. Many investigators have linked HLA polymorphisms to disease resistance or susceptibility (10). Particularly, cervical cancer appears to be associated with specific HLA combinations or HLA-DQA1 variants (11) in a virus-host model. Genotyping of this HLA locus could permit the early identification of women at high risk for viral infection and cervical disease development, representing a risk factor. This fast and economical method is accurate and easy to interpret and well suited for routine clinical or research purposes.

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