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High Resolution Allotyping of Four Alleles of HLA-DRB1*01 Group in Iranians Using Reverse-SSOPH Assay in Comparison with DNA Sequencing and PCR-SSP

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Abstract: Described here an amplification-based Sequence Specific Oligonucleotide Probe Hybridization (SSOPH) assay for high resolution allotyping of four HLA DRB1*01 group alleles. A region within exon 2 of HLA DRB1 gene was amplified by using group specific biotin-labeled primers. The amplicons were then hybridized to immobilized oligonucleotide probes that were specific for HLA DRB1*01 group alleles (HLA DRB1*0101, *0102, *0103 and *0104). The Hybridized amplicons were detected by an enzymatic-colorimetric reaction. One hundred and fifty DNA samples were tested by this method, in parallel with PCR-SSP and DNA sequencing for demonstrating the accuracy of the Reverse Dot Blot (RDB). Results suggested that PCR-RDB is a rapid, accurate and cost effective method for high resolution HLA molecular typing in comparison to PCR-SSP and DNA sequencing.

Key words: PCR-RDB, high resolution, HLA typing, HLA DRB1*01 group alleles

INTRODUCTION

The human Major Histocompatibility Complex (MHC) is considered within about 4 Mbp of DNA on the short arm of chromosome 6 at 6p21.3 (Marsh, 2007). A number of PCR-based methods for HLA class II typing have been emerged in recent years in order to provide a clear picture of the molecular basis of allelic polymorphism; although, they have their own limitations (Moalic and Ferec, 2005; Altermann *et al.*, 2006; Itoh *et al.*, 2006). More recently a new methodology based on DNA microarray has developed to identify Single Nucleotide Polymorphism (SNPs) encoded within the highly polymorphic MHC genes and allows simultaneous analysis of many SNPs in DNAs from a large number of individuals, in a single experiment (Jiang *et al.*, 2006; Palmisano *et al.*, 2005). PCR-Sequence Specific Priming (PCR-SSP) (Savelkoul *et al.*, 1995; Downing *et al.*, 2004; Davidson and Poulton, 2001) and Sequence Specific Oligonucleotide Probe Hybridization (SSOPH) (Fig. 1) are the most common techniques that are being used for molecular HLA typing

(Saiki *et al.*, 1986; Buhler *et al.*, 2002; Buyse *et al.*, 1997; Williams, 2001). The use of molecular HLA typing in organ transplantation improves the quality of such transplantations (Tiercy, 2002) and entails better clinical consequences and more precise compatibility testing. The final HLA typing for matching potential bone marrow donors and recipients should be done with a method that allows high-resolution typing (Sheldon and Poulton, 2006; Flomenberg *et al.*, 2004; Kögler *et al.*, 2005). In order to obtain such a high resolution (Oudshoorn *et al.*, 2007), methods such as PCR-SSP or PCR-SSOP can be used but these procedures require large number of primers and probes. In this study, attempts were made to benefit from the advantages of both SSP and SSOP procedures (Middleton *et al.*, 2000). For this purpose, HLA DRB1 alleles were divided to several allelic groups and pairs of primers were designed for amplification of each group of alleles (Doxiadis and Claas, 2003; Kawai *et al.*, 1996). Subsequently, 5'-amino oligonucleotide probes were used for determination of each allele. Here the first allelic group (HLA DRB1*01) was selected for group specific

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amplification and each of the alleles (*0101, *0102, *0103, *0104) were tracked by means of specific probes. Selection of these alleles were considered with high frequency of them within DRB1*01 allele group in Iranian population except for DRB1*0104 which is our aim about it was to find at least one sample owing to rare frequency of this allele in the world (Amirzargar *et al.*, 2001; Collins *et al.*, 2000).

MATERIALS AND METHODS

Samples: Three DNA samples resulted from B cell lines of the 10th International Histocompatibility Workshop (IHW) that had been characterized by DNA sequencing of HLA-DRB1 locus were received from Anthony Nolan Research Institute (London, UK) as a source of reference DNA to validate the method (Table 1). We had no DNA sample of HLA-DRB1*0104; therefore, we prepared a synthetic standard DNA according to sequence of *0104 that is available on the following web site: <http://www.ebi.ac.uk/imgt/hla> (Robinson *et al.*, 2003). One hundred and fifty individuals referred to Iranian Blood Transfusion Organization (I.B.T.O, Tehran, Iran) in 2006 for taking HLA typing test. After informed consent was given, blood samples were collected from unrelated healthy bone marrow donors. The IBTO records were used to verify patient's data and the study protocol conforms to the ethical guidelines of the 1975 declaration of Helsinki as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran.

HLADRB1 typing by PCR-SSP with commercial kits:

DNA samples from 150 unrelated healthy bone marrow donors were extracted using DNA isolation kit for mammalian blood (Roche, Germany) and typed by using two commercially available Kits. Firstly, they were typed by traditional kit (Protrans GmbH, Ketsch, Germany) that

was generic for DRB1 locus in order to determination of HLA-DRB1*01 allele group. Then, these samples were typed by a high resolution kit (Dynal Biotech, USA) that was allele specific for DRB1*01 group.

PCR amplification and labeling: Group-specific amplification of DRB1*01 allelic group was performed using primers specific (M.W.G biotech, Germany) (Table 2) for a region within exon 2 of HLA DRB1 gene (Allen *et al.*, 1998).

We used 50-100 µg mL⁻¹ of genomic DNA (standard control samples HLA-DRB1 *0101, *0102, *0103, *0104), 1 unit Taq DNA polymerase, 0.2 mM dNTP, 2 mM MgCl₂ and 1×PCR buffer (50 mM KCl, 20 mM Tris-HCl with pH = 8.4) for PCR reactions in total volume of 25 µL. Reactions were performed in a programmable thermocycler (Eppendorf, Germany) at 94°C for 30 sec, at 62°C for 20 sec, at 72°C for 40 sec for 35 cycles. The last cycle was followed by an extension time at 72°C for 10 min. Finally, PCR products were detected using 1.2% agarose gel electrophoresis (Allen *et al.*, 1998).

Specificity of primers: For evaluation of the specificity, not target DNAs, notably non HLA-DRB1*01 samples, were used to confirm the correct function of primers.

Covalent coupling of oligonucleotide probes to the membrane:

Amino-linked Oligonucleotide probes (5'-NH₂ oligoprobes) (M.W.G biotech) with a C6 spacer were diluted to the optimized concentration about 2 pmol in 0.5 M NaHCO₃. The sequence of one of which was complementary to a common region in all of this group alleles and other four were specific to *0101, *0102, *0103 and *0104 alleles (Table 3). A Biodyne C membrane (Pall Biosupport, NJ) was activated by incubation in 16% (W/V) EDC {1-Ethyl-3-(Dimethylamino propyl) Carbodiimide hydrochloride} for 10 min at 25°C. Following a brief wash with deionized water for about 5 to 10 min, 10 µL of each diluted oligonucleotide was applied in a dot by using a miniblotted (Biometra, Germany) and incubated 2 min at room temperature. Then, the oligonucleotide solutions were removed from the membrane by aspiration

Table 1: IHW cell line DNA used to evaluate accuracy

Samples	Locus and specificities
KAS116	HLA-DRB1*0101
MZ070782	HLA-DRB1*0102
TER-ND	HLA-DRB1*0103

Table 2: PCR primers used to amplify HLA DRB1*01 allele group

Primer	Sequence	Length	T _m
F. primer	5'-TTTCTTGTGGCAGCTTAAGTTTGAA-3'	25 bp	58°C
R. primer	5'-Biotin-ACTCGCCGCTGCACTGTGAAGCT-3'	23 bp	66°C

Table 3: Oligonucleotide probes used to detection different HLADRB1*01 group alleles

Name	Sequence	Specificity	T _m
RBM1	5'-NH ₂ -TGGAACAGCCAGAAGGAC-3'	*0101 and *0103	56°C
RBS1	5'-NH ₂ -ACGGGGCTGTGGAGAG-3'	*0102	56.9°C
RBN2	5'-NH ₂ -CTGGAAGACGAGCGGG-3'	*0103	56.9°C
RBT1	5'-NH ₂ -GGGGTTGTGGAGAGCTT-3'	*0104	55.2°C
RBG2	5'-NH ₂ -TGAGACACAACTACGGG-3'	*0101 to *0104	56°C

in which way they were applied. The membrane was inactivated by incubation in 0.1 M NaOH for 10 min at room temperature, followed by a brief wash with deionized water for about 5 to 10 min. The membrane was incubated in 20 mM EDTA for 20 min at room temperature and stored at 4°C (Schollen *et al.*, 1997).

Sequence specific oligonucleotide probe hybridization using reverse dot blotting: The membranes were prehybridized individually in 4×SSPE buffer (1×SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM Na₂EDTA at pH 7.4) and 0.5% SDS at 46°C for 30 min. Ten microliters of amplified DNA was denatured by boiling for 5 min, each added to a membrane strip and hybridized for 50 min at 46°C. After hybridization the membranes were washed at 46°C for 10 min in 2× SSPE buffer and 0.1% SDS. The membranes were then blocked with 0.1% BSA and after that detection was done using Streptavidin-alkaline phosphatase conjugate and NBT/BCIP (Roche, Germany) color detection substrate. The color was developed in the dark at room temperature for 20-40 min. The strips were air dried and photographed.

Optimizing probe concentration: RBG2 oligoprobe was applied to the Biodyne C membrane in a dilution series ranging from 20 to 0.1 pmol to optimize the probe concentration for immobilization on the membranes.

RESULTS

PCR amplifications: PCR amplifications with HLA DRB1*01 group specific primers were performed on standard samples and some samples not belonging to HLADRB1*01 allele group. The samples belonging to one of the DRB1*01 group alleles were specifically detected by gel electrophoresis (Fig. 1-3).

Reverse SSOPH of standard DNAs: Results of reverse dot blot detection system for known standard samples (IHW cell line DNA) of HLA-DRB1 *0101, *0102, *0103 and *0104 alleles are shown in Fig. 4. The samples were tested in duplicated. RBG2 probe was placed in the set of oligoprobes in order to hybridize to every DR*01 subspecificities, thus providing a positive control for the efficiency of hybridization.

Probe concentration: Hybridization efficiency was studied using different amounts of membrane-immobilized Oligonucleotide. As demonstrates (Fig. 5), we were able to obtain an optimal signal by immobilizing as little as 0.1 pmol of Oligonucleotide.

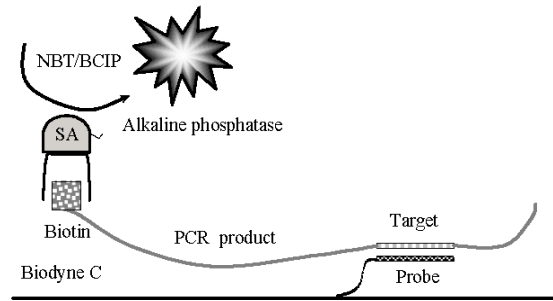


Fig. 1: Schematic of the reverse dot blot assay using immobilized probes, - SA: Streptavidin

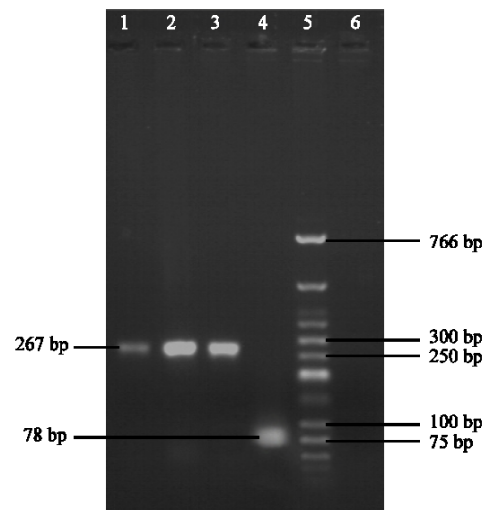


Fig. 2: Gel electrophoresis of HLA-DRB1*01 specific PCR products. Lane 1-4 are standard DNA samples corresponding respectively to HLA-DRB1*0101, *0102, *0103, *0104. Lane 5 is DNA size marker (Roche, Germany) and Lane 6 is control negative

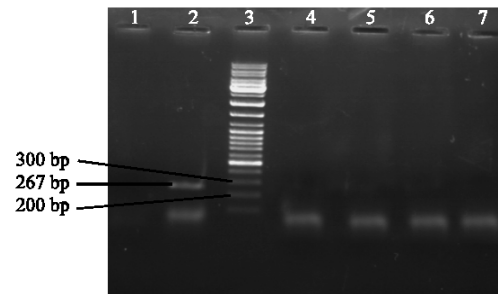


Fig. 3: Electrophoresis of confirmation of primers specificity by non HLA-DRB1*01 DNA samples. Lane 1 is control negative; Lane 2 is HLA-DRB1*01 sample; Lane 3 is DNA size marker (Fermentas, Lithuania). Lanes 4-7 are non HLA-DRB1*01 samples

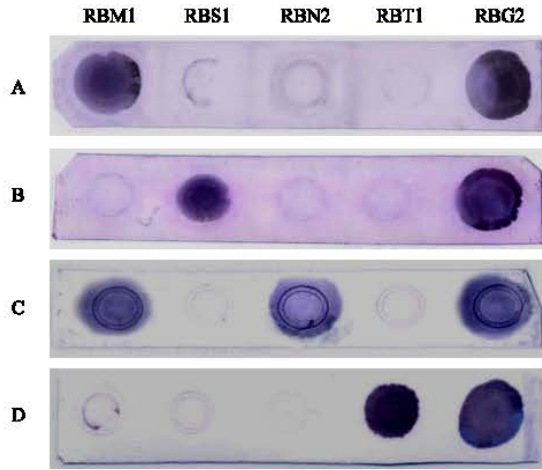


Fig. 4: Reverse SSOPH strips after hybridization and color development. Hybridization probes complementary to the most common HLA-DRB1*01 group alleles were fixed to these reverse dot blot strips. On each filter strip five probes were attached and RBG2 was control probe. Samples: A, HLA-DRB1*0101; B, HLA-DRB1*0102; C, HLA-DRB1*0103; D, HLA-DRB1*0104

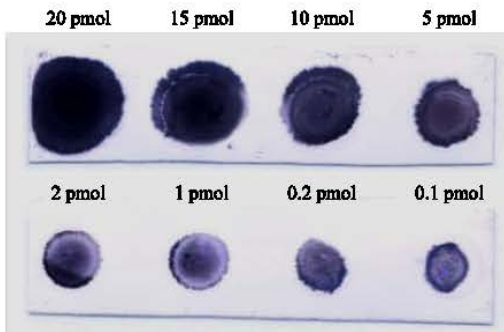


Fig. 5: Hybridization test of oligonucleotide probe RBG2 with varying concentrations. Probe (NH₂-5' TGC AGA CAC AAC TAC GGG-3') was diluted as indicated, subsequently immobilized on the membrane and hybridized to PCR amplified HLA-DRB1*01

Reverse SSOPH in comparison with PCR-SSP and DNA sequencing: HLADRB1*01 allelic type was determined in collaboration with IBTO on 150 blood samples of unrelated healthy bone marrow donors by SSP method by using commercial kit. Out of 150 samples, 12 samples were typed as HLA DRB1*01 group, out of which 8 samples were *0101 type and 4 samples were *0102 type. These samples also were typed by DNA sequencing, the most

Table 4: Comparison of three main techniques in high resolution HLA-DRB*01 allotyping

Sample type	Methods		
	RDB	PCR-SSP	DNA sequencing
HLA-DRB1*0101	8/12	8/12	8/12
HLA-DRB1*0102	4/12	4/12	4/12
HLA-DRB1*0103	0/12	0/12	0/12
HLA-DRB1*0104	0/12	0/12	0/12

precise mean of high resolution typing and these results also corresponded to reverse dot blot and PCR-SSP methods (Table 4).

DISCUSSION

The HLA Class II loci are highly polymorphic and classification of these polymorphism is important in cases of organ transplantation, paternity, forensics and other areas (Gerlach, 2002). Different methods are currently being used for genetic typing of class II HLA. The development of an HLA genotyping strategy is complicated due to the relatively large number of polymorphic sites in these genes. There are several HLA genotyping methodologies available, all with some advantages and disadvantages. Direct sequencing of HLA genes is likely to be superior to other genotyping methods when high resolution is needed (Van der Vlies *et al.*, 1998). However, disadvantages include low throughput, time intensive scoring of alleles and ambiguities in assignment of some heterozygous allele combinations. Sequence-Specific Priming (SSP) for HLA genotyping has been used in the clinical laboratory where typing is performed on a relatively small number of samples and is often the method used to confirm results from other methods (Feolo *et al.*, 2001). Another procedure for detection of genetic polymorphism is based on sequence specific oligonucleotide probes (SSOPH) (Kennedy *et al.*, 1995). This technique requires a large number of hybridization reactions to distinguish between allelic combinations, but is capable of high throughput and has the potential to identify new HLA alleles. In this respect, we used group specific primers for amplification of an allelic group and specific probes for identification of related alleles in that particular group. Thus we only used limited number of probes that were specific for the allelic group amplified by PCR. Different methods were used to increase sensitivity of detection to an extent that the results could be detected obviously. The major change that was introduced for this purpose was to design special probes that had a C₆ spacer linked to a solid support in order to overcome problems related to positional limitation of probe designing which otherwise could lead to a very weak results. If probes are bound by

UV or heat, they will be attached in random fashion to the membrane and sometimes to each other, making it more difficult to determine consistent or optimal hybridization conditions (Zhang *et al.*, 1991; Saiki *et al.*, 1989). Therefore, we applied a covalent site-specific attachment of such oligonucleotide probes to membranes which had been designed with nearly equal melting temperatures (T_m) for specifically attachment to target sequences. On the other hand, temperature equilibration and a uniform exposure of the membrane to conjugate and substrate are critical for obtaining optimal results (Gold, 2003). In this study all of the data obtained from reverse-SSOPH procedure were in accordance with results obtained by standard SSP method and direct sequencing, suggesting that this assay is reliable for the molecular HLA typing. In conclusion, PCR-Reverse SSOPH that enables allotyping of HLA DRB1*01, can be applied for high resolution typing of all other allelic groups of this locus and other loci. This procedure is rapid, sensitive, specific and simple to perform. It can be used for first line typing of HLA genes on large number of samples in bone marrow registry centers (Hurley *et al.*, 1999; Anasetti *et al.*, 2001). This assay can also be used for high resolution HLA typing before transplantation from unrelated donors.

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