Original Article

HLA-DR Typing by Polymerase Chain Reaction with Sequence- Specific Primers Compared to Serological typing

M.Adib *, M. Yaran **, A. Rezaie *, G. Solgi *

ABSTRACT

Background: Considering the role of HLA matching in transplant outcome, the quality of HLA-DR typing is clearly an important issue. In recent years, serological methods have been replaced with DNA based typing methods. The main objective of this study was to compare HLA-DR typing data obtained from existing serologic method with data obtained by the new PCR-SSP method.

Methods: 55 peripheral blood samples were collected from randomly selected individuals who were referred to the transplantation laboratory of Isfahan, in Aliasghar Hospital, and were typed for HLA-DR antigens by both methods. HLA-DR typing by serologic method was performed using 30 different antisera and for PCR-SSP method, specific primers were used for HLA-DRB1*01-10(except DR6, 8, 10), and also for HLA-DR52, and DR53. After DNA extraction, 13 pairs specific primers were used for each sample separately and PCR reaction were done. In this study, the third intron of DR locus was used as internal positive control. After PCR amplification, products of reaction electrophoresis was performed on 2% agarose gel, and after taking photo of gel, interpretation and comparison of results were performed.

Results: The results of 31 samples (56.3%) corresponded completely to serological method, 12 samples (22%) were assigned heterozygous in serology and homozygous in molecular typing, 7 samples (12.7%) were heterozygous in both methods but different in one allele. 2 samples (3.6%) were homozygous in serology and heterozygous in molecular typing, and also one sample (1.8%) was homozygous in both methods but so that in serology DR14, and in molecular typing DR11 were assigned. And finally 2 samples from 55 (3.6%) were not detectable in serological method.

Conclusion: The typing data obtained from the conventional and the new methods were compared. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. The results indicated that the DNA based method had more sensitivity, accuracy, and resolving power than serologic typing methods.

Key words: HLA-DR, PCR-SSP, serological typing.

Archive of SID

The major histocompatibility complex (MHC) is a genetic region which was initially defined by the rejection of skin grafts in genetically incompatible mouse strains. In humans, the MHC, known as human leukocyte antigens (HLA), are separated into HLA-I and HLA-II based on their structure, tissue distribution and function [1]. The genes that encode HLA-I (HLA-A, B, C) and HLA-II (HLA-DR, DP, DQ) molecules are the most polymorphic genetic system in the human genome, so much that some loci (e.g. HLA-B or DR) have more than 300 alleles [2]. Initially, genetic variations at these loci were analyzed by serologic typing using reagents derived from sera of multiparous women or individuals who had received multiple blood transfusion. Nowadays, with respect to the extensive variations of HLA molecules and scarcity of mono specific antibodies for detection of each antigen, HLA-I and HLA-II can be typed at DNA level with more accuracy (fewer errors) and more precision (more discriminating) by molecular techniques compared to serologic typing [3,4]. DNA based approaches to HLA typing have proven to confer significantly greater sensitivity, accuracy, and

* Department of Immunology, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran.
** Biotechnology Laboratory, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran.
Correspondence to: Dr.Minoo Adib, Department of Immunology Medical School, Isfahan University of Medical Sciences, Isfahan, Iran.
This study was supported financially by a grant from Isfahan University of Medical Sciences (Grant No. 79295).

www.SID.ir

resolving power than serologic typing methods [5].

Molecular techniques demonstrate that extensive HLA polymorphism, already recognized by classic serologic approach is very underestimated [6, 7]. Based on the available database of class-I and class-II allelic sequence diversity, a variety of PCR- based typing such as Restriction fragment length polymorphism (RFLP), Sequence specific primers (SSP), Sequence specific oligonucleotide probes (SSOP), Single strand conformational polymorphism (SSCP), Sequence based typing (SBT), and DNA chip have been developed and applied for clinical HLA typing [8].

Amplification with sequence specific primers (SSP) is a widely used molecular technique that can be optimized for high throughput low to medium resolution typing [9]. The aim of the present study was to perform HLA-DR typing by PCR-SSP and serologic methods and to compare the results.

Materials and Methods

55 different peripheral blood samples were drawn from randomly selected donor and recipient individuals who had been referred to the transplantation laboratory of Aliasghar hospital in Isfahan from March 2002 to March 2003.

HLA typing for DR specificities were performed by microlymphocytotoxicity method [10], using 30 different commercialy obtained antisera.

To determine HLA-DR alleles by PCR-SSP method, sequence specific primers, corresponding to the serologically defined alleles [HLA-DRB1*01-10 (except DR6, 8, 10), DR52 and DR53] were obtained (Geneset oligoes france) and used for experiments.

PCR-SSP

Genomic DNA was extracted from peripheral blood sample by modified salting out method [11]. In brief, 2 ml of whole blood was mixed with 8 ml of triton lysis buffer 1 (0.32M Sucrose, 5mM MgCl2.6H2O, 12mM Tris-HCl, pH 7.5, 1%V/V Triton X-100). Leukocytes and nuclei were spun down (3500g, 5min), the pellet was washed with dH20 and then resuspended in 0.9 ml of lysis buffer 2 (0.375M NaCl, 0.12M EDTA, pH 8.0), 25 µl SDS 10%, and 0.22 ml NaClO4 (4M) and was shaken vigorously , spun down (13000g, 5 min) and subsequently salted out using a saturated NaCl solution. DNA in the supernatant was precipitated with 99.5% ethanol. Finally, DNA pellet was dissolved in 100 µl of ddH2O.

After quantitation of DNA by UV spectrophotometer, 100ng of genomic DNA was used for each 20 µl PCR reaction. For HLA-DR low resolution typing by PCR-SSP , 14 separate reactions were done per sample: ten for assigning HLA-DRB1* 01, 03, 04, 07, 09, 11, 15, 16; three for assigning DR52 and DR53; and finally a negative control (in which DNA was replaced by H2O) was included in each sample. With regard to low prevalence of DR6, 8, and 10 in Isfahan population, primers for these alleles were not used[12].

The PCR reaction mixtures consisted of PCR buffer (50mM KCl, 1.5mM MgCl2, 10mM Tris-Hcl, pH 8.3), 0.01% w/v gelatin, 200µM of dNTP mix , 1µM of allele or group specific DRB primers[13], 0.2µM of control primers (amplified the third intron of DRB1genes), 1 unit of Taq polymerase (sinagen), and 100 ng of genomic DNA. PCR amplification was carried out in a PCR set (Techne- Genius). After initial denaturation at 95^0C for 5 minutes, DNA was amplified by 30 three temperature cycles; denaturation at 95^0C for 20 sec , primer annealing at 61^0C for 30 sec , and extension at 72^oC for 30 sec .

Absence or presence of PCR products was visualized by agarose gel electrophoresis. After addition of 5 µl loading buffer (40% w/v sucrose, 0.25% Bromophenol Blue) , the PCR reaction mixtures were loaded in 2% agarose gel , and then gels were run for 15-20 minutes at 10 V/cm in 0.5x TBE (89mM Tris base, 89mM Boric acid, 2mM EDTA pH 8.0). After staining with ethidium bromide (1µg/ml H2O) for 15 min, gels were examined under UV illumination and documented by photography [13].

Results

At first, in order to confirm the precision of PCR-SSP for DR typing, a family with four children where typed with PCR-SSP method, thereby the transmision of genes from parents to siblings was determined. The obtained results and inheritance of haplotypes corresponded with mandelian traits. Subsequently, DR typing was performed on prepared samples from transplantation laboratory. In this study, discrepancy of results between serology and PCR-SSP consisted of: 12 of 55 samples (22%) were assigned heterozygous in serological typing and homozygous in PCR-SSP, 2 of 55 samples (3.6%) were homozygous in

Archive of SID

www.SID.ir

HLA-DR typing by PCR compared to serological typing serological typing and heterozygous in PCR-SSP, Also 7 samples (12.7%) were heterozygous in both methods but were different in one allele. Just one case (1.8%) was homozygous in both methods, which was determined as DR11 in PCR-SSP and DR14 in serology methods.

Two samples (3.6%) were not assigned in serology, whereas all of 55 individuals were typed by PCR-SSP. Finally, results of 31 samples (56.3%) corresponded in both methods (table 1). The distribution of discrepancy among HLA-DR alleles in PCR and serology are illustrated in table 2.

In this study Sensitivity and specificity of serology method in compare with PCR-SSP were 77% and 92%, respectively. Also positive predictive value and negative predictive value were 80% and 93%, respectively. 30 of 55 samples were typed on three separate occasions by PCR-SSP and interpreted blindly. The reproducibility between the repeated typings was 100%.

Table1. Percentage and the type of discrepancy for DRB1/B3/ B4 alleles between serology and PCR-SSP methods.

PCR-SSP vs. Serology	No. of cases	%
Complete Matched Sample	31	56.3
Antigen Vs Blank	2	3.6
Antigen Vs Antigen	7	12.7
Blank Vs Antigen	12	22.0
Homozygote (DR11Vs DR14)	1	1.8
Typed Vs Undetectable	2	3.6
Total	55	100

Antigen Vs Blank: One allele had not been assigned in serology (False negative).
Antigen Vs Antigen: An allele was incorrectly assigned in serology.
Blank Vs Antigen: The sample, basically is homozygous and therefore the second allele is false Positive in serology.

Discussion

The HLA antigens are major barriers in transplantation of organ and tissue between individuals [14]. Recent data analysis about the role of HLA matching in renal transplantation has consistently shown a stepwise decrease in graft survival rate with increasing antigen mismatch [4,15].

The importance of matching for class I &II alleles in clinical outcome of unrelated Bone Marrow Transplantation (BMT) have been well established.

Table 2. Distribution of discrepancy among HLA-DR alleles in PCR-SSP and serology.

HLA-DR specificity	number	Discrepancy Rate
HLA-DRB1*01	3	33.0%
HLA-DRB1*15	15	20.0%
HLA-DRB1*16	2	0.0%
HLA-DRB1*03	21	42.0%
HLA-DRB1*04	23	28.0%
HLA-DRB1*11	33	24.0%
HLA-DRB1*07	4	0.0%
HLA-DRb1*09	1	100.0%
HLA-DRB3	43	9.0%
HLA-DRB4	26	25.0%

Indeed, even a minute of difference by a single amino acid may cause acute rejection [9].

Considering the role of HLA matching in transplantation outcome, the quality of HLA-DR typing is clearly an important issue. The initial study of Opelz et al, indicated that among 107 serologically HLA-A, B, DR compatible transplants, 29 had broad mismatched DR antigen, when retyped using RFLP technique. Transplants with these undetermined mismatched DR had graft survival rates that were 22% lower than those who were confirmed as HLA matched [15].

The surprisingly high rate of discrepancies in typing of healthy bone marrow of volunteer donors shows the importance of DNA based molecular typing [16].

PCR-based methods of HLA-I and II typing have been developed. These are simple, rapid, highly informative, automated methods, and can be carried out at either intermediate or high levels of allelic resolution in clinical diagnostic settings as well as for research studies [15,17,18]. Already many clinical transplantation laboratories have implemented the DNA based techniques for HLA-DR typing [6]. The current results provide further motivation for implementation of molecular HLA typing in clinical histocompatibility testing [19]. The comparison of DNA typing and serology indicates that DNA matched grafts survive better than serologically matched grafts, probably reflecting differences in accuracy (fewer error for DNA typing) rather than greater discrimination. In these comparisons, the DNA typing was carried out at

Archive of SID

www.SID.ir

a low to intermediate level of resolution (does not distinguish all alleles) [20-22].

In present study, the great differences between serology and PCR-SSP consisted of: in 22% of samples, an additional allele was assigned in serology (false positive). Also 12.7% of the differences resulted from incorrect detection in serology rather than being non assigned (table 1). However, the precision and accuracy of both methods were compared only based on the detection of serologically defined HLA-DR alleles, and indeed, the used primers were specific for determination of these alleles. Despite of these, the difference between the results of two methods was 43.7% that indicated the higher error in serology or more accuracy in PCR-SSP for DR typing; Because in molecular typing such as PCR-SSP, the factors including quantity, quality, and viability of the cells, lack of monospecific antiserum, difference in time and temperature of incubation, precision in reading of microplates, and etc, that are variable in serology, do not affect the PCR-SSP [7,23]. Results of both methods about DR3 allele show statistically significant difference (P=0.035) because of the lack of monospecific antiserum for DR3 and also mixture of DR5 and DR11 antiserums with DR3 antiserum in the utilized microplates.

Although the difference of results about DR9 between both methods was 100%, but since this state was seen in only one case, it was not statistically significant (P=1.000). Also two undetectable cases, and incorrect serological typing in one case (for DRB1 alleles) were not acceptable results for transplant patients.

The same studies have indicated 10- 57% difference rate between serology and DNA-based typing methods[20-24]. With respect to these results, exploitation of HLA-DNA typing in histocompatibility testing is inevitable. Including the DNA extraction procedure (by NaClO4) that was used in this research, the PCR-SSP, was performed in 3 hours. Also there was not need for post PCR specificity steps. Therefore the presence or absence of PCR products that is the principle of PCR-SSP, was visualized by gel electrophoresis. In emergency conditions such as cadaver kidney transplantation, PCR-SSP in compare with serology and other molecular typing, is preferable because of its simplicity and rapidity [13].

Briefly, other merits of PCR-SSP consist of: a) wider variety of samples can be used. This is because the viability of the cells or expression of the relevant HLA on the cell surface isn't required, as it is in serological typing [1,7,23]; b) It can be carried out at either intermediate or high levels of allelic resolution [17,20] ; c) Simplicity in preparation of required substances and their stability [15,17]. d) No false positive and false negative results [18,25]. e) Without any need for the amplification of pseudogenes [26] ; And finally high performance, more accuracy (fewer errors), much more precise (more discriminating), and automability of this technique [1,7,23].

Acknowledgment

We would like to thank Dr. G Opelz from the transplantation center, University of Heidelberg, Germany, for providing HLA-DR primers and his kind technical assistance.

Archive of SID

References

1. *Erlich HA, Opelz G, Hansen J. HLA DNA typing and transplantation. Immunity 2001 Apr; 14(4):347-56.*
2. *Turner D, Akpe S, Brown J, Brown C, McWhinnie A, Madrigal A, Navarrete C. HLA-B typing by reference strand mediated conformation analysis using a capillary-based semiautomated genetic analyzer. Hum Immunol 2001 Apr; 62(4):414-8.*
3. *Anderson G. Evolution of the human HLA-DR reigion. Frontiers in Bioscience. 3th Edition. 1998; July 27: 739-745.*
4. *Zafar MN, Ahmed N, Naqvi A, Rizvi A. Impact of DNA typing on a living-related donor renal transplant program. Transplant Proc 1999 Dec; 31(8):3335.*
5. *Petersdorf EW, Mickelson EM, Anasetti C, Martin PJ, Woolfrey AE, Hansen JA. Effect of HLA mismatches on the outcome of hematopoietic transplants. Curr Opin Immunol 1999 Oct; 11(5):521-6.*
6. *Grams SE, Wu J, Noreen HJ, Mangaccat J, Cognato MA, Johnson S, Segall M, Williams TM, Begovich AB. Three new DP alleles identified in a study of 800 unrelated bone marrow donor-recipient pairs. Tissue Antigens 2001 Oct; 58(4):272-5.*

www.SID.ir

7. *Adorno D, Canossi A, Papola F, et al. Comparison between HLA class I PCR-ARMS and serological typing in cadaveric kidney transplantation.Transplantation Proceeding 1997; 29: 1423-1425.*
8. *Marsh SG. Nomenclature for factors of the HLA system, update March 2000. WHO Nomenclature Committee for Factors of the HLA System. Tissue Antigens 2000 Jul; 56(1):103-4.*
9. *Sayer D, Whidborne R, Brestovac B, et al. HLA-DRB1 DNA sequencing based typing: an approach suitable for high throughput typing including unrelated bone marrow registry donors. Tissue Antigens 2001 Jan; 57(1):46-54.*
10. *Terasaki T. Histocompatibility Testing. Copenhagen: Munsgaard; 1980: p290-298.*
11. *Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989 Apr 11; 17(7):2503-16.*
12. *Adib M, Abkar-Shahnazar E. Gene and antigen frequency of HLA class II (DR and DQ) in 300 cases in isfahan. Isfahan Res Med Sci J 1997; 1: 7-9.*
13. *Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 1992 May; 39(5):225-35.*
14. *Opelz G, Wujciak T, Dohler B. Is HLA matching worth the effort? Collaborative Transplant Study. Transplant Proc 1999 Feb-Mar; 31(1-2):717-20.*
15. *Michael Cecka J. The Role of HLA in Renal Transplantation. Human Immunol 1997; 56: 6-16.*
16. *Noreen HJ, Yu N, Setterholm M, et al. Validation of DNA-based HLA-A and HLA-B testing of volunteers for a bone marrow registry through parallel testing with serology. Tissue Antigens 2001 Mar; 57(3):221-9.*
17. *Chen M, Duffy BF, Mohanakumar T. New aspects in Histocompatibiliy Testing. Laboratory Medicine Newsletter 1996 March; 4(3).*
18. *Olerup O, Zetterquist H. HLA-DRB1*01 subtyping by allele-specific PCR amplification: a sensitive, specific and rapid technique. Tissue Antigens 1991 May;37(5):197-204.*
19. *Opelz G, Mytilineos J, Scherer S, Trejaut J, Dunckley H, Chapman J, Fisher G, et al. Analysis of Discrepancy between serological and DNA-RFLP Typing for HLA-DR in kidney graft recipients. Transplant Proc 1992; 24: 2478.*
20. *Schaffer M, Olerup O. HLA-AB typing by polymerase-chain reaction with sequence-specific primers: more accurate, less errors, and increased resolution compared to serological typing. Tissue Antigens 2001 Nov;58(5):299-307.*
21. *Cermakova Z, Kolarikova H. Serological versus molecular HLA-DR typing of cadaveric donors in conditions of Regional Tissue Typing Laboratory. Bone Marrow Transplant 1998 Dec;22 Suppl 4:S47-8.*
22. *Opelz G, Mytilineos J, Scherer S, Schwarz V. Clinical implications of DNA typing in organ transplantation. The Collaborative Transplant Study. Transplant Proc 1997 Feb-Mar;29(1-2):1524-7.*
23. *Ota M, Katsuyama Y, Liu CY, Arakura A, Fukushima H. Validation of HLA-DR locus typing in forensic specimens by combining PCR-SSP with PCR-RFLP. J Forensic Sci 1997 Sep;42(5):929-34.*
24. *Mytilineos J, Christ U, Lempert M, Opelz G. Comparison of typing results by serology and polymerase chain reaction with sequence-specific primers for HLA-Cw in 650 individuals. Tissue Antigens 1997 Oct;50(4):395-400.*
25. *Savelkoul PH, de Bruyn-Geraets DP, van den Berg-Loonen EM. High resolution HLA-DRB1 SSP typing for cadaveric donor transplantation. Tissue Antigens 1995 Jan;45(1):41-8.*
26. *Knipper AJ, Hinney A, Schuch B, Enczmann J, Uhrberg M, Wernet P. Selection of unrelated bone marrow donors by PCR-SSP typing and subsequent nonradioactive sequence-based typing for HLA DRB1/3/4/5, DQB1, and DPB1 alleles. Tissue Antigens 1994 Nov;44(5):275-84.*

Archive of SID

www.SID.ir