Comparative study between serological and PCR-SSP methods to determine class II HLA molecules

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ABSTRACT. The direct detection of HLA polymorphism was an extremely difficult task until a short time ago, mainly for Class II alleles. It has become easier due to Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) technique. Serological and PCR-SSP methods were compared when antigens HLA-DR and –DQ were typed in healthy donors with homozygous phenotype (blanks) and in patients who, for some reason, could not be typed by serological method. This comparative study showed that 21% of healthy donors showing blanks by serological method had their alleles defined by molecular method (PCR-SSP) and that 17% of renal patients with neither proper quantity nor quality of B lymphocytes to define their Class II HLA antigens were all defined by the PCR-SSP method. Molecular typing was adequate for patients with low B lymphocytes quantity and quality. Phenotypic homozygosis index for HLA-DR decreased, HLA-DQ1 splits alleles were better defined, and rare associations between DRB1 and DQB1 were established.

Key words: HLA, microlymphocytotoxicity, PCR-SSP.

RESUMO. Estudo comparativo entre os métodos sorológico e PCR-SSP na determinação das moléculas HLA de classe II. A detecção direta do polimorfismo HLA, que até recentemente era uma tarefa difícil, principalmente para os antígenos HLA de classe II, foi facilitada com o advento da reação de polimerização em cadeia (PCR) na definição de alelos por biologia molecular. Os métodos, sorológico e reação de amplificação em cadeia utilizando-se primer de seqüência específica (PCR-SSP), foram comparados na tipificação dos antígenos/alelos HLA-DR e -DQ em doadores saudáveis com o fenótipo de homozigose ("blanks") e em pacientes que, por alguma razão, não foi possível tipificá-los pelo método sorológico. Este estudo demonstrou que 21% dos doadores, apresentando "blanks" pelo método sorológico, tiveram seus alelos definidos pelo método molecular e 17% dos pacientes renais, que não apresentavam quantidade e qualidade adequada de linfócitos B para a definição de seus antígenos HLA de classe II, todos foram definidos pelo método PCR-SSP. A tipagem molecular mostrou-se adequada para tipificar pacientes com baixa quantidade e qualidade de linfócitos B circulantes, diminuiu o índice de homozigose fenotípica para HLA-DR, possibilitou melhor definição dos "splits" de HLA-DQ1 e também evidenciou o estabelecimento de associações raras entre DRB1 e DQB1.

Palavras-chave: HLA, microlinfocitotoxicidade, PCR-SSP.

Introduction

Major Histocompatibility Complex (HLA system in humans) is characterized by an extensive degree of polymorphism. During the last 40 years HLA typing to detect this polymorphism was undertaken by the serological method (Terasaki and Mc Clelland, 1964), better known as the complement-dependent cytotoxicity assay (CDC). This method is still used in some HLA laboratories especially for typifying Class I antigens (HLA-A, -B). However, the isolation of B cells is necessary to type Class II antigens (HLA-DR, -DQ). Until recently such isolation has been achieved by different methods, but typing results were not always reliable (Verduyn *et al*., 1993; Thorogood *et al*., 1993). However, the relevance of HLA-DR for solid organ transplantation and stem cells transplantation could be established even with such low reliability degree (Dupont and Hansen, 1978).

A correct assignment of HLA antigens is very important, since inadequate HLA-matching of patient-donor pairs is associated with rejection in

kidney transplants, and rejection or graft-versus-host disease in allogenic bone marrow transplants (Opelz *et al*., 1991; Kerman *et al*., 1993). Recent progress in assigning Class II HLA alleles by techniques involving DNA analysis indicates that Class II HLA typing by serology may not be sufficiently reliable (Mytilineos *et al*., 1990; Olerup *et al*., 1993).

Quality and availability of antiserum reacting with certain HLA-DR types are among the limitations in Class II HLA typing by serology. Similarly, B lymphocytes obtained by venipuncture in patients may not satisfy minimal criteria needed for successful Class II HLA typing techniques.

Molecular biological methods seemed to be a powerful tool to detect Class II HLA alleles as a substitute for serological methods**.** Molecular HLA typing, particularly for HLA-DR genes, has shown that there is more polymorphism than can be detected by serological typing methods (Olerup and Zetterquist, 1992; Bryan *et al*., 1993; Otten *et al*., 1995; Tiercy, 2002; Velickovic *et al*., 2004; Balas *et al*., 2004).

Serology and PCR-SSP for HLA -DR and -DQ typing have been compared in health donors, showing "blanks" in their typing results, and in renal patients who, for some reason, could not be serologically typed.

Material and methods

Subjects

Samples were obtained from 106 individuals (88 healthy donors and 18 renal patients).

Typing HLA molecules by the serological method

HLA serological typing method, called Complement Dependent Cell Microlymphocytotoxicity (Terasaki and McClelland, 1964), employs lymphocytes from peripheral blood since they are a good source of HLA molecules. HLA typing was performed by Special Monoclonal Typing Tray Class II, Locus: DR/DQ (SMDR72, One Lambda Inc., Canoga Park, CA).

Typing HLA Class II molecules by the molecular method

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes by Qiamp spin Column (Quigen Inc. Chatswcth, CA), according to manufacturer's recommendations. DNA concentration was adjusted to 100 ng/mL in bi-distilled water.

Amplification reaction of genomic DNA

Amplification was performed by Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) (Newton *et al*., 1989) employing Micro SSP^{TM} DNA (One Lambda, Inc. Canoga Park, CA, USA). Assay was performed for DNA typing of alleles *HLA DRB1*, 3*, 4*, 5** and *DQB1**. Pre-optimized primers for the amplification of Class II HLA genes in the reaction were distributed in wells of PCR plate for later addition of DNA samples, Taq polymerase **(**Invitrogen, Life technologies**)** and dNTP-buffer mix. After amplification, electrophoresis in agar gel 2.5% was undertaken, at 150 volts, for approximately 5 minutes. A pair of specific primers amplifying a limited region of ß-human globulin gene, present in all DNA samples, was used as a positive control of reaction.

Statistical analysis

Statistical analysis was performed using simple kappa coefficient (Landis and Koch, 1977).

Results

Serological and molecular PCR-SSP methods were compared for HLA typing in 106 individuals (88 health donors and 18 renal patients). Figure 1 demonstrates that 62% of health donors had concordant results with HLA-DR typing. However, a difference in HLA-DR typing was found in 21% of health donors, whereas in 18 patients (17%) the HLA-DR typing was possible only by PCR-SSP (kappa coefficient = 0.60).

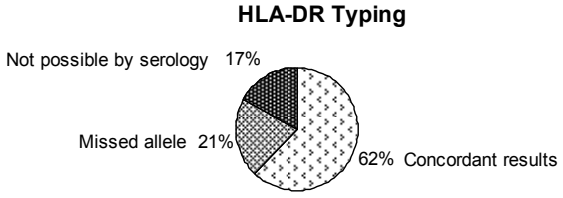

Figure 1. Analysis of HLA-DR typing results generated by serologic and Polymerase Chain Reaction-Sequence Specific Primer methods in 106 individuals.

Comparison of HLA-DR typing by serology and by PCR-SSP showed that 22 healthy donors had discrepancies in serologic "blanks" which turned out to be definable alleles by PCR-SSP (Table 1).

Table 1. Discrepancies in HLA-DR typing results between serology and Polymerase Chain Reaction-Sequence Specific Primer.

No. of cases	Serology	PCR-SSP
2	DR 17,X	*DRB1*03, *13*
4	DR4,X	*DRB1*04, *13*
3	DR11,X	*DRB1*11, *13*
1	DR7,X	*DRB1*07, *13*
1	DR1,X	*DRB1*01, *04*
1	DR11,X	*DRB1*08, *11*
1	DR1,X	*DRB1*01, *11*
1	DR15,X	*DRB1*13, *15*

1	DR7,X	*DRB1*04, *07*
1	DR11,X	*DRB1*03, *11*
1	DR15,X	*DRB1*15, *16*
1	DR2,X	*DRB1*13, *15*
1	DR13,X	*DRB1*04, *13*
1	DR4,X	*DRB1*04, *12*
1	DR11,X	*DRB1*04, *11*

No: number; PCR-SSP: Polymerase Chain Reaction-Sequence Specific Primer.

Results showed that among 88 health donors, 21 (23.8%), HLA-DQ1 splits (HLA-DQ5 and HLA-DQ6) were better defined by PCR-SSP methodology (Table 2).

Table 2. Discrepancies in HLA-DQ typing results between serology and Polymerase Chain Reaction-Sequence Specific Primer.

No. of cases	Serology	PCR-SSP
1	DQ1, X	*DQ3, 5*
5	DQ1, X	*DQ5, 6*
3	DQ1, X	*DQ6, X*
4	DQ1, X	*DQ5, X*
1	DQ1, 7	*DQ3, 6*
5	DQ1, 2	*DQ2, 6*
1	DQ1, 3	*DQ3, 5*
1	DQ1, 3	*DQ3, 6*

No: number; PCR-SSP: Polymerase Chain Reaction-Sequence Specific Primer.

HLA typing analysis also showed that PCR-SSP method enabled the establishment of rare associations between *loci* DRB1 and DQB1, such as *DRB1*13 and DQB1*05; DRB1*04 and DQB1*06*, which had not been previously determined by the serum methods.

Discussion

Since HLA-DR and DQ typing results obtained by serologic and molecular PCR-SSP methods were compared, we may conclude that PCR-SSP method was more accurate and reliable than serology for DRB1 and DQ typing. Our research showed that 38% of individuals had their typing HLA improved when PCR-SSP method was used. About 21% of the above percentages were healthy donors that had missed alleles and 17% were renal patients that could not be typed by serology due to low viability and small quantity of cells**.** In the case of HLA-DQ typing, 23.8% of healthy donors could have their HLA-DQ1 better defined and consequently the matching process in the bone marrow receptors could be improved also.

A reliable HLA typing method to select suitable kidney and bone marrow donors is obviously very important. Missed alleles and incorrect assignments by serology may result in incorrect donor selection (Opelz *et al*., 1991**;** Wu *et al*., 2002; Noreen *et al*., 2001). Patients undergoing haemodialysis for a long period of time could have their B-cell viability decreased and the separation of these cells from blood are affected, making the output small (Mytilineos *et al*., 1990; Usberti *et al*., 2002). In current study 17% of renal patients were injured due to delay or inconclusive HLA-DR typing. Consequently they could not be in the waiting list to apply for a cadaver donor. No such problem exists in the case of DNA typing since DNA samples may be easily obtained from fresh blood buffy-coat. HLA-DR typing by the PCR-SSP method may be generated from all patients regardless the quantity or quality of B-cells in their circulation system. In addition, when the number of circulating nucleated cells is too low to get enough DNA**,** it may be extracted from any nucleated cell source (Otten *et al*., 1995; Hayney *et al*., 1996).

Conclusion

The results showed that when the serological and molecular methods are compared for HLA typing, moderate agreement was found (kappa coefficient = 0.60). Nevertheless, molecular method proved to be useful in determining HLA-DR molecules, mainly for patients in which certain problems in serological tests may be expected. PCR-SSP method may detect HLA Class II alleles that could be easily lost or incorrectly determined by the serological technique.

References

BALAS, A. *et al.* Sequencing of two new HLA class II alleles: DRB3∗ 0218 and DQB1∗ 030202. *Tissue Antigens*, Copenhagen, v. 63, p. 614-6, 2004.

BRYAN, C.F. *et al*. Impact of HLA-DR typing by polymerase chain reaction amplification with DR[beta]1 sequence specific primer on cadaveric renal allocation . *Transplant Proc.***,** New York, v. 25, p. 3060, 1993**.**

DUPONT, B.; HANSEN, J.A. Donor selection for bone marrow transplantation: The predictive value of HLA-D typing for MLR compatibility between unrelated individuals. *Transplant Proc.*, New York, v. 10, p. 53, 1978.

HAYNEY, M.S. *et al*. A noninvasive 'swish and spit' method for collecting nucleated cells for HLA typing by PCR in population studies. *Hum. Hered*., New York, v. 46, p. 108-11, 1996.

KERMAN, N. A. *et al*. Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. *N. Engl. J. Med*., Boston, v. 328, p. 593-602, 1993.

LANDIS, J.R.; Koch, G.G. An application of hierarchical Kappa-type statistics in the assessment of majority agreement among multiple observers. *Biometrics*, Washington, DC, v. 33 n. 2, p. 363-374, 1977.

MYTILINEOS, J. *et al*. Comparison of RFLP-DR beta and serological HLA-DR typing in 1500 individuals. *Transplantation*, Baltimore, v. 50, p. 870-3, 1990.

NEWTON, C.R. *et al*. Analysis of any point mutation in DNA. The amplification refractory mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.,* London, v. 17, p. 2503- 2516, 1989

NOREEN, H. J. *et al*. Validation of DNA-based HLA-A and HLA-B testing of volunteers for a bone marrow

registry through paralleled testing with serology. *Tissue Antigens*, Copenhagen, v. 57, p. 221-9, 2001**.**

OLERUP, O. *et al*. HLA-DR by PCR amplification with sequence-specific primers (PCR-SSP) in 2hous. *Tissue Antigens*, Copenhagen, v. 41, p. 119-34, 1993.

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***,** Copenhagen, v. 30, p. 225, 1992.

OPELZ, G. *et al*. Survival of DNA HLA-DR typed and matched cadaver kidney transplants. *Lancet*, London, v. 338, p. 461-3, 1991.

OTTEN, H. G. *et al*. Serology versus PCR-SSP in typing for HLA-DR and HLA-DQ: a practical evaluation. *Tissue Antigens***,** Copenhagen, v. 45, p. 36-40, 1995.

TERASAKI, P. I.; Mc CLELLAND, J. D. Microdroplet assay of human serum cytotoxins. *Nature*, London, v. 204, p. 998, 1964.

THOROGOOD, J. *et al*. Survival of serologically HLA typed and matched cadaver kidney transplants and patients: Influence of serological retyping of donors. *Transplant Proc.*, New York, v. 25, p. 3051-3052, 1993.

TIERCY, J. M. Molecular basis of HLA polymorphism: implication in clinical transplantation. *Transpl. Immunol.*, Dunton Green, v. 9, p. 173-80, 2002.

USBERTI, M. *et al*. Effects of a vitamin E-bonded membrane and of glutathione on anemia and erythropoietin requirements in haemodialysis patients. *J. Nephrol.*, Rome, v. 15, p. 558-64, 2002**.**

VELICKOVIC, Z. M. *et al*.; Identification of two novel HLA class II alleles DQB1∗ 030503 and DRB1∗ 0447. *Tissue Antigens*, Copenhagen, v. 63, p. 93-5, 2004.

VERDUYN, W. *et al.* Biotinylated sequence-specific oligonucleotides: Comparison to serological HLA-DR typing of organ donors in Eurotransplant. *Hum. Immunol*., New York, v. 37, p. 59-67, 1993.

WU, D. L. *et al*. Comparative analysis of serologic typing and HLA-II typing by micro-PCR-SSP. *Di Yi Jun Yi Da Xue Xue Bao*, Guangzhou Shi Tonghe Zhen, v. 22, p. 247-9, 2002.

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