Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9291
ISSN on-line: 1807-8648
Doi: 10.4025/actascihealthsci.v36i1.15575

Discrepancies in HLA typing: use of different methods to confirm doubtful or inconclusive results

Rejane Cristina Ribas-Silva[1], Adriana Danmvolf Ribas[2], Luciana Borges Giarola[3], Marina Raduy Botelho[4] , Marco Antonio Braga[4] and Sueli Donizete Borelli[4*]

*[1]Faculdade Integrado de Campo Mourão, Campo Mourão, Paraná, Brazil. [2]Centro Universitário de Maringá, Maringá, Paraná, Brazil. [3]Faculdade Ingá, Maringá, Paraná, Brazil. [4]Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brazil. *Author for correspondence. E-mail: sueliborelli@gmail.com*

ABSTRACT. The major histocompatibility complex (MHC) is a set of genes found on the short arm of chromosome 6. MHC molecules in human beings are known as human leukocyte antigens (HLA). HLA polymorphism can be determined by serological and molecular typing methods, which may yield discordant results. The present analysis performed HLA typing of samples with discordant results by PCR-SSP and PCR-SSO, so that typing discrepancies could be clarified. The cross-sectional study analyzed 33 samples from individuals included in an HLA-disease association study. Discrepant alleles were observed in 6 of 33 samples. Discordant samples were retyped using One Lambda Micro SSP™, Dynal RELI™ SSO and Luminex™ SSO assays for HLA class I (HLA-A, HLA-B) and class II (HLA-DRB1) molecules. The three methods produced concordant results after HLA retyping. Human error occurred in interpreting the initial results, which led to discrepancies in the results obtained. The participation of experienced professionals and the availability of at least two different methods to confirm doubtful or inconclusive results are mandatory for effective HLA typing.

Keywords: HLA antigens, histocompatibility testing, polymerase chain reaction.

Discrepâncias na tipagem HLA: diferentes métodos para confirmar resultados duvidosos ou inconclusivos

RESUMO. O complexo principal de histocompatibilidade (MHC) é um conjunto de genes encontrados no braço curto do cromossomo 6. Em humanos, as moléculas de MHC são conhecidas como antígenos leucocitários humanos (HLA). Polimorfismo HLA pode ser determinado por métodos de tipagem sorológica e molecular que são susceptíveis de produzir resultados discordantes. Este estudo teve como objetivo realizar a tipagem HLA de amostras com resultados discordantes por PCR-SSP e-SSO e para esclarecer discrepâncias de digitação. Este estudo transversal analisou 33 amostras de indivíduos incluídos em um estudo de associação HLA-doença. Alelos discrepantes foram observados em seis das 33 amostras. Amostras discordantes foram retyped usando One Lambda Micro SSP™, Dynal RELI™ SSO Luminex e ensaios ™ SSO para HLA de classe I (HLA-A, HLA-B) e classe II (HLA-DRB1) moléculas. Todos os três métodos apresentaram resultados concordantes após HLA redigitação. Houve erro humano na interpretação dos resultados iniciais o que levou a uma discrepância entre os resultados obtidos. Concluiu-se que a participação de profissionais experientes e com a disponibilidade de pelo menos dois métodos diferentes para confirmar os resultados duvidosos ou inconclusivos são essenciais para a tipagem de HLA eficaz.

Palavras-chave: antígenos HLA, teste de histocompatibilidade, reação em cadeia da polimerase.

Introduction

The major histocompatibility complex (MHC) is a set of genes found on the short arm of chromosome 6. MHC molecules in human beings are known as human leukocyte antigens (HLA) or the HLA system (PEAKMAN; VERGANI, 1999).

Determination of HLA molecules is of great importance in transplants since HLA compatibility is crucial to the success of transplantation, in spite of the use of immunosuppressive drugs to reduce the possibility of rejection (GALLARDO, 2010; GJERTSON, 2002; OPELZ et al., 2010).

HLA polymorphism can be determined by serological (TERASAKI; McCLELLAND, 1964) and molecular (LAVANT et al., 2011) methods. Molecular typing, currently the method of choice in histocompatibility laboratories, provides better resolution than serological typing.

The present study was conducted when discrepancies in HLA typing results were observed

during an HLA-disease association study conducted in the same laboratory and by independent research groups, hereinafter referred to as group X and group Y. Group X conducted an association study using the Micro SSP™ assay (One Lambda®) for HLA-A, -B and -DRB1 typing. After some time, group Y initiated another HLA-disease association study and used Dynal RELI™ SSO for HLA typing. During HLA typing, samples from some patients used by both research groups (groups X and Y) showed discrepant HLA typing results. Owing to the above, the present analysis performed HLA typing of samples with discordant results, using three different methods so that typing discrepancies could be clarified.

Material and methods

Genomic DNA was extracted from 33 peripheral blood mononuclear cell (PBMC) samples by the salting-out method. The participants were informed of the objectives of the study and signed a consent form. The present research was approved by the Research Ethics Committee (CNS/196 no. 311/6) of the State University of Londrina, Londrina, Paraná State, Brazil, and conducted in accordance with the provisions of the Declaration of Helsinki.

PCR-SSP typing (Micro SSP™, One Lambda®): amplification was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP). Primer pairs were designed to make perfect matches only with a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly matched primer pairs caused the amplification of target sequences (i.e., a positive result), while mismatched primer pairs did not result in amplification (i.e., a negative result). After the PCR process, the amplified DNA fragments were separated by 2.5% agarose gel electrophoresis and revealed by staining with SYBR Safe® DNA gel stain (Invitrogen Corp.) and by exposure to ultraviolet light. Interpretation of PCR-SSP results was based on the presence or absence of a specific amplified DNA fragment on the gel, and this information was then manually transferred to worksheets (interpretation tables) to define HLA haplotypes (Figure 1).

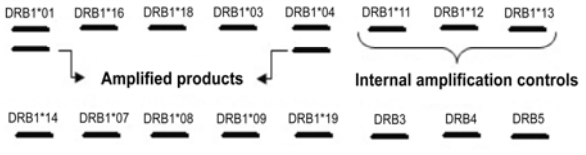

Figure 1. Illustration of typing of HLA-DRB1 alleles or group of alleles using sequence-specific primers (SSP). According to amplified products, sample HLA typing is HLA-DRB1*01, DRB1*04. DRB4.

PCR-SSO typing (Dynal RELI™ SSO): an array of sequence-specific oligonucleotide (SSO) probes was immobilized on a nylon membrane. The biotin-labeled amplicons were bound to the membranes that contained a complementary target sequence. After hybridization, a thorough wash removed unbound amplicons to ensure reaction specificity. A colorimetric reaction was produced by addition of the peroxidase-labeled conjugate (streptavidin-horseradish peroxidase, SA-HRP) for visualization of the amplicon-specific probe. The addition of hydrogen peroxide (H_2O_2) and tetramethylbenzidine (TMB) as a substrate caused the formation of a complex that turned blue in the presence of SA-HRP. The color of the test probes was compared to the color intensity of control probes. Results were interpreted according to the manufacturer's instructions (DYNAL, 2009).

PCR-SSO typing (One Lambda LABType® using Luminex™): biotinylated primers were used. The PCR-amplified product was denatured and allowed to rehybridize with probes bound to microspheres (beads) that are part of the Luminex multianalyte system. Beads were associated with a fluorescence-based color, and each bead color showed a unique oligonucleotide probe corresponding to an HLA allele or group of HLA alleles. After hybridization, the probes that hybridized with the DNA under analysis were labeled by an R-Phycoerythrin-conjugated Strepavidin (SAPE) solution. Fluorescent-labeled beads and SAPE contents bound to probes were read on the LABScan 100 flow cytometer. HLA Fusion™ was used for data analysis.

All procedures were carried out according to the manufacturer's recommendations and interpreted under the supervision of qualified professionals.

Results and discussion

Discrepant HLA typing results were detected in 6 of 33 samples (18.2%) analyzed by two different PCR-SSO techniques and one PCR-SSP technique. (Table 1).

Two discrepancies were detected in the HLA-A locus. The first sample was typed as HLA-A*24, A*30 by PCR-SSP and as HLA-A*24, A*32 by PCR-SSO. The second sample was typed as HLA-A*03, A*30 by PCR-SSP and as HLA-A*02, A*30 by PCR-SSO.

Two discrepant results were also detected in the HLA-B locus. The same sample was typed as HLA B*27, B*27 by PCR-SSP and as HLA-B*27, B*51 by PCR-SSO. The second sample was typed as HLA-B* 07, B*45 by PCR-SSP and as HLA-B* 07, B*50 by PCR-SSO.

Table 1. Results of HLA-A, -B and -DRB1 retyping of 6 discrepant samples by PCR-SSP and PCR-SSO.

Samples	MICRO SSP™	Dynal RELI™ SSO	Luminex™ SSO
Sample 1	HLA-A*30,68	HLA-A*30,68	HLA-A*30,68
	HLA-B*38,44	HLA-B*38,44	HLA-B*38,44
	*HLA-DRB1*09,14*	*HLA-DRB1*09,09*	*HLA-DRB1*09,09*
Sample 2	HLA-A*03,24	HLA-A*03,24	HLA-A*03,24
	HLA-B*44,51	HLA-B*44,51	HLA-B*44,51
	*HLA-DRB1*01,14*	*HLA-DRB1*01:02,13*	*HLA-DRB1*01:02,13*
Sample 3	HLA-A*23,29	HLA-A*23,29	HLA-A*23,29
	*HLA-B*07,45*	*HLA-B*07,50:02*	*HLA-B*07,50:02*
	HLA-DRB1*11,14	HLA-DRB1*11,14	HLA-DRB1*11,14
Sample 4	HLA-A*02,02;	HLA-A*02,02;	HLA-A*02,02;
	HLA-B*27,27;	HLA-B*27,51;	HLA-B*27,51;
	HLA-DRB1*04,15	HLA-DRB1*04,15	HLA-DRB1*04,15
Sample 5	HLA-A*03,30	HLA-A*02,30	HLA-A*02,30
	HLA-B*18,51	HLA-B*18,51	HLA-B*18,51
	HLA-DRB1*07,13	HLA-DRB1*07,13	HLA-DRB1*07,13
Sample 6	*HLA-A*24,30*	*HLA-A*24,32*	*HLA-A*24,32*
	HLA-B*44,57	HLA-B*44,57	HLA-B*44,57
	HLA-DRB1*01,11	HLA-DRB1*01,11	HLA-DRB1*01,11

PCR = polymerase chain reaction; SSO = sequence-specific oligonucleotide; SSP = sequence-specific primers; *The discordant locus of each sample is shown in italic.

Two discrepancies were observed in the HLA-DRB1* locus. One sample was typed as HLA-DRB1*01, DRB1*14 by PCR-SSP and as HLA-DRB1*01, DRB1*13 by PCR-SSO. The other sample was typed as HLA-DRB1*09, DRB1*14 by PCR-SSP method and as HLA-DRB1*09, DRB1*09 by PCR-SSO.

Analysis of the results revealed that the discrepancies between HLA typing methods of repeated samples were due to problems of interpretation and human error, and did not involve any failure in accuracy of the techniques employed. Discrepant results between different techniques should be analyzed carefully. In addition to possible HLA typing errors, which can be solved by using other typing methods, discrepancies may translate into the identification of novel HLA alleles (LONGHI et al., 2003).

Although the use of a particular technique is dependent on equipment availability, reproducibility of methods, number of samples to be processed, technical expertise of the staff, financial resources and other factors, laboratories can use a combination of methods to better suit their needs (MIDDELTON, 2005).

Most studies report on the differences between serological and molecular typing methods (TAN; TANG, 1999, TORIO et al., 2002, TIERCY et al., 2003; BORELLI et al., 2004). Few authors have addressed discrepancies in typing results between molecular methods, perhaps due to an underlying fear of admitting their own errors or because they believe that disclosure is unimportant.

It is the authors' conviction that reporting this experience is important to point out errors that may occur in any histocompatibility laboratory, especially in those where graduate students may be involved in the performance of techniques and interpretation of HLA typing.

Human error in PCR-SSP typing technique has been reported in the interpretation of agarose gel bands, in which faint bands were either ignored or unnoticed. Furthermore, human error was found to have occurred during the transferring of results observed on the gel to the worksheets used in the interpretation of results.

According to Tan et al. (2000), HLA typing by PCR-SSP proved to be an accurate, reliable and well-reproducible technique. However, the method is considered too difficult to use for clinical assays involving large-scale screening. Frequently it requires human interpretation without the aid of software systems, which increases the susceptibility to human error.

Although the use of PCR-SSO typing techniques does not eliminate the possibility of interpretation errors, Dynal RELI™ and Luminex™ SSO assays have their own software systems as a tool to aid in the interpretation of results. Therefore, the availability of at least two different HLA typing methods is essential for the clarification of doubtful results and for internal quality control in histocompatibility laboratories.

Further, Sequence-based typing (PCR-SBT) is another, more accurate methodology by which the locus is amplified and sequenced to determine the specific allele (Torres, 2010). The method is actually more suitable for high-resolution characterization, allowing the identification of alleles and revealing incompatibilities that were not identified by serological or molecular methods with low or 'average' resolution (PEREIRA et al., 2010).

Another important factor is the training of professionals and academics by company representatives, who can provide detailed information on the different typing methods. There is a need for professionally technical and scientific knowledge gained by identifying the presence of rare alleles, linkage disequilibrium, ambiguities, or homozygous typing. It is also highly important that the analysis of results be supervised by trained professionals, in order to minimize or avoid possible technical failures or interpretation errors.

Conclusion

Training of professionals and students by company representatives, who can provide detailed information on the different typing methods, is of paramount importance. It is also highly important that the analysis

of results be supervised by trained professionals, to minimize or avoid possible technical failures or interpretation errors. In case of doubt regarding HLA typing results, it is recommended that both methods be carried out or that more accurate techniques, such as PCR-SBT, be employed.

References

BORELLI, S. D.; SOUZA, D.; FREITAS, K. F. F.; BOTINI, F. F.; MELO, F. C.; BEDENDO, J. Comparative study between serological and PCR-SSP methods to determine class II HLA molecules. **Acta Scientiarum. Health Sciences**, v. 26, n. 2, p. 337-340, 2004.

DYNAL. **Dynal RELI user's manual**. Available from: <http://www.siercheng.com/UploadFile/20078223335148 4.pdf>. Access on: Sep. 22, 2009.

GALLARDO, D. Clinical impact of HLA disparities in transplants from unrelated donors. **Methods and Findings in Experimental and Clinical Pharmacology**, v. 32, Suppl. A, p. 27-29, 2010.

GJERTSON, D. W. A multi-factor analysis of kidney regraft outcomes. **Clinical Transplants**, v. 2002, p. 335-349, 2002.

LAVANT, E. H.; AGARDH, D. J.; NILSSON, A.; CARLSON, J. A. A new PCR-SSP method for HLA DR-DQ risk assessment for celiac disease. **Clinica Chimica Acta**, v. 412, n. 9-10, p. 782-84, 2011.

LONGHI, E.; FRISON, S.; POLI, F.; GABOARDI, D.; FERRIOLI, G.; SCALAMOGNA, M. Identification of a novel HLA-DRB1 allele: DRB1*1353. **Tissue Antigens**, v. 61, n. 2, p. 177-178, 2003.

MIDDELTON, D. HLA Typing from Serology to Sequencing Era. **Iranian Journal of Allergy, Asthma and Immunology**, v. 4, n. 2, p. 53-66, 2005.

PEREIRA, N. F.; OLIVEIRA, D. C. M.; TORRES, M.; RODRIGUES, C. A.; ALENCAR, I. S. B.; SALOMÃO, I.; MAUAD, M. A.; COLTURATO, V. A. R.; BOUZAS, L. F. S.; MORAES, M. E. Seleção de doador de medula óssea ou sangue periférico. **Revista Brasileira de Hematologia e Hemoterapia**, v. 32, n. 7, p. 3-5, 2010.

OPELZ, G.; SÜSAL, C.; RUHENSTROTH, A.; DÖHLER, B. Impact of HLA Compatibility on lung transplant survival and evidence for an HLA restriction phenomenon: A collaborative transplant study report. **Transplantation**, v. 90, n. 8, p. 912-17, 2010.

PEAKMAN, M.; VERGANI, D. **Imunologia básica e clínica**. Rio de Janeiro: Guanabara Koogan, 1999.

TAN, J.; TANG, X. DNA typing for HLA-A by polymerase chain reaction with sequence-specific primers. **Hua Xi Yi Ke Da Xue Xue Bao**, v. 30, n. 2, p. 133-137, 1999.

TAN, J.; TANG, X.; XIE, T. Comparison of HLA class I typing by serology with DNA typing. **Zhonghua Yi Xue Za Zhi**, v. 80, n. 3, p. 187-189, 2000.

TERASAKI, P. I.; McCLELLAND, J.D. Microdroplet assay of human serum cytotoxins. **Nature**, v. 204 n. 4962, p. 998-1000, 1964.

TIERCY, J. M.; STADELMANN, S.; CHAPUIS, B.; GRATWOHL, A.; SCHANZ, U.; SEGER, R. A.; FAVERI, G. N.; KERN, M.; MORELL, A.; SCHWABE, R.; SCHNEIDER, P. Quality control of a national bone marrow donor registry: results of a pilot study and proposal for a standardized approach. **Bone Marrow Transplantation**, v. 32, p. 623-627, 2003.

TORIO, A.; MOYA-QUILES, M. R.; MURO, M.; MONTES-ARES, O.; ONTANON, J.; MINGUELA, A.; MARIN, L.; ALEMANY, J. M.; SANCHEZ-BUENO, F.; ALVAREZ-LOPES, M. R. Discrepancies in HLA-C typing in transplantation: comparison of PCR-SSP and serology results. **Transplantation Proceedings**, v. 34, n. 1, p. 419-420, 2002.

TORRES, M. A. Human leukocite antigens (HLA): informações básicas para um hematologista. In: HAMERSCHLAK, N. (Org.). **Manual de hematologia**, Barueri: Manole, 2010. cap. 55, p. 425- 430.

Received on December 20, 2011.
Accepted on August 28, 2012.