

SHORT COMMUNICATION

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Identification of HLA Class I Misreads/Dropouts Using Serological Typing, in Comparison with DNA-based Typing

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ABSTRACT

Serology and DNA techniques are employed for Human Leukocyte Antigen (HLA) typing in different transplant centers. Results may not always correlate well and may need retyping with different technique. All the patients (with aplastic anemia, thalassemia, and immunodeficiency) and their donors, requiring HLA typing for bone marrow transplant were enrolled in the study. Serological HLA typing was done by complement-dependent lymphocytotoxicity while DNA-based typing was done with sequence specific primers (SSP). Serology identified 167 HLA A and 165 HLA B antigens while SSP in same samples identified 181 HLA A and 184 HLA B alleles. A11 and B51 were the commonest antigens/alleles by both methods.

There were a total of 21 misreads and 32 dropouts on serology, for both HLA A and B loci with HLA A32, B52 and B61 being the most ambiguous antigens.

Inherent limitations of serological techniques warrant careful interpretation or use of DNA-based methods for resolution of ambiguous typing.

Keywords: Ambiguous antigens; Complement dependent lymphocytotoxicity (CDC); DNA based HLA typing; Human leukocyte antigen typing; Sequence specific primers (SSP)

INTRODUCTION

Human Leukocyte Antigen (HLA) typing for the purpose of matching between bone marrow transplant (BMT) recipient and donor remains the cornerstone for successful transplant outcome.¹ Although individual transplant centers may have different matching requirements, it is usually preferable to have matching not below 6/8 loci considering haplotype pairs of HLA

A, B, C, and DRB1.² Fully matched recipients of peripheral blood stem cells carry a five-year-survival rate of 71.3% in Pakistan.³

Several HLA typing strategies are employed for routine typing of donor recipient pairs. Broadly these have been classified into serological- and DNA-based techniques. Serology is based on complement dependent lymphocytotoxicity (CDC) technique, while DNA-based typing relies on sequence specific primers (SSP), sequence specific oligonucleotide probes (SSOP), and sequence based typing (SBT).⁴ In recent years, DNA based typing has outdone serology in multitude of ways. It employs synthesized products and, thus, does not require viable sera/cells obtained

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from multiparous women or multiply transfused persons, as in CDC. It overcomes problems of poor cell viability or poor surface HLA expression, thus helping to confirm or refute phenotypic homozygosity.⁵ Perhaps the best utility of DNA-based typing lies in determining the HLA alleles more accurately, which is especially important in unrelated bone marrow transplants. Problems of cross-reactive groups encountered in serology have been overcome now. Sequence based typing allows high resolution typing along with determination of new alleles. The higher cost of DNA-based methods still hampers their wide scale application for routine HLA typing, but with the introduction of next generation sequencing (NGS) technologies, these assays will become affordable in coming years.⁶

In our laboratory, we routinely type bone marrow transplant pairs for HLA class I initially by serology. If they are matched, full HLA typing is performed using SSP which is a DNA based technique. However, sometimes, during the initial serological workup, ambiguities arise and we have to resort to SSP. In this study, we compared the results of our serology with SSP as the standard method, with the aim of identifying problematic antigens that are frequently misread on serology. We also identified some antigens that were not readable on serology but were detected on SSP.

MATERIALS AND METHODS

This observational comparative study was carried out from January 2014 to December 2015 in Immunology Department of Armed Forces Institute of Pathology, after approval by institutional ethical committee (No. Imm/16/01). All the subject pairs (patients with aplastic anemia, thalassemia, and immunodeficiency along with their donors, requiring HLA typing for bone marrow transplant) referred for HLA typing, who were either serologically matched at HLA class I locus or whose serological results required DNA-based typing for resolution of ambiguities, were included in this study.

HLA class I typing by serology was carried out by CDC technique using the commercial prepared plates (One Lambda Inc, USA). Lymphocyte separation was done by density gradient centrifugation of heparinized sample. Separated B and T lymphocytes were added to plates containing antibodies against predefined antigens. This resulted in immune complex formation.

Complement addition resulted in its activation by previously formed immune complexes and cell lysis. Lysed cells took up the stain eosin and appeared dark under phase contrast microscope. Two observers read plates independently and antigen assignment was done with agreement. For SSP typing, DNA was extracted using commercial kit (Qiagen Germany) according to the manufacturer's instructions. Concentration of DNA was adjusted to 100 ng/ul. The DNA purity was tested by measuring absorbance of DNA solution at 260/280 nm. It was stored at -20°C till further analysis. HLA gene amplification at A and B loci was carried out with commercial kit (One Lambda Inc, USA) that uses sequence specific primers, specifically targeting exon 2 and 3 of both loci which harbor most of the polymorphism observed.⁷ Amplification was done by polymerase chain reaction (PCR) in Eppendorf thermal cycler. Internal control for PCR was used as included in the kit. The product was analyzed on 2% agarose gel using bromophenol blue dye as indicator.⁸ Number of wells on agarose gel showing amplification band of the product were recorded and allele assignment was done using the worksheet. Appearance of internal control primer band was pre-requisite for successful PCR.

The assigned alleles were recorded in Microsoft Excel (Microsoft Excel 2011, version 14.1.3, USA) against their serological counterparts. Antigens misread by serology were assigned to "antigenic misreads" and antigens not detected at all by serology to "antigenic dropouts". Identification of antigenic misreads and antigenic dropouts were carried out manually in Microsoft Excel (Microsoft Excel 2011, version 14.1.3, USA) by identifying serology vs SSP mismatches.

RESULTS

Over the study period of two years, 102 (72 males, 30 females) samples necessitated HLA typing by SSP method, either because donor and patient were fully matched by serology or serological ambiguities required further clarification by different DNA based technique. Mean age of study subjects was 12±11.6 years.

Figure 1 shows total number of antigens detected by serology compared to total number of alleles detected by SSP for both HLA A and B loci. It is evident that SSP was more sensitive by identifying 181 alleles for HLA A and 184 alleles for HLA B in contrast to

serology that identified 167 and 165 antigens for HLA A and HLA B, respectively.

Figures 2 and 3 show antigens/alleles distribution for HLA A and B loci, respectively; with A11/A*11 and B51/B*51 being the commonest in study population, when detected by both techniques.

Figure 4 shows total number of misreads and dropouts on serology that were later detected on SSP, for both HLA A and B loci. HLA A locus showed

fewer misreads/dropouts as compared to HLA B.

Table 1 shows distribution of HLA alleles that have been either misread or altogether dropped out on serology, which were then detected by SSP. HLA A32 was the most ambiguous antigen that was twice misread and four times dropped out altogether by serology. Similarly B52 was three times misread while B61 was dropped out five times on HLA B locus.

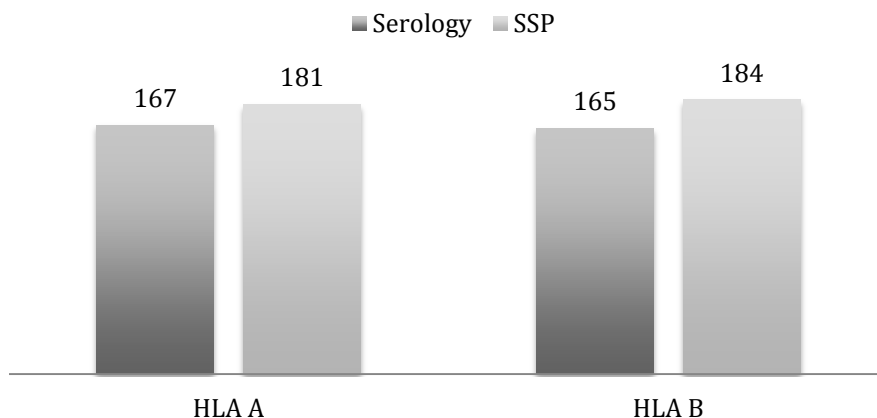


Figure 1. Total number of antigens detected vs number of alleles detected using serology and sequence specific primers (SSP) techniques, respectively.

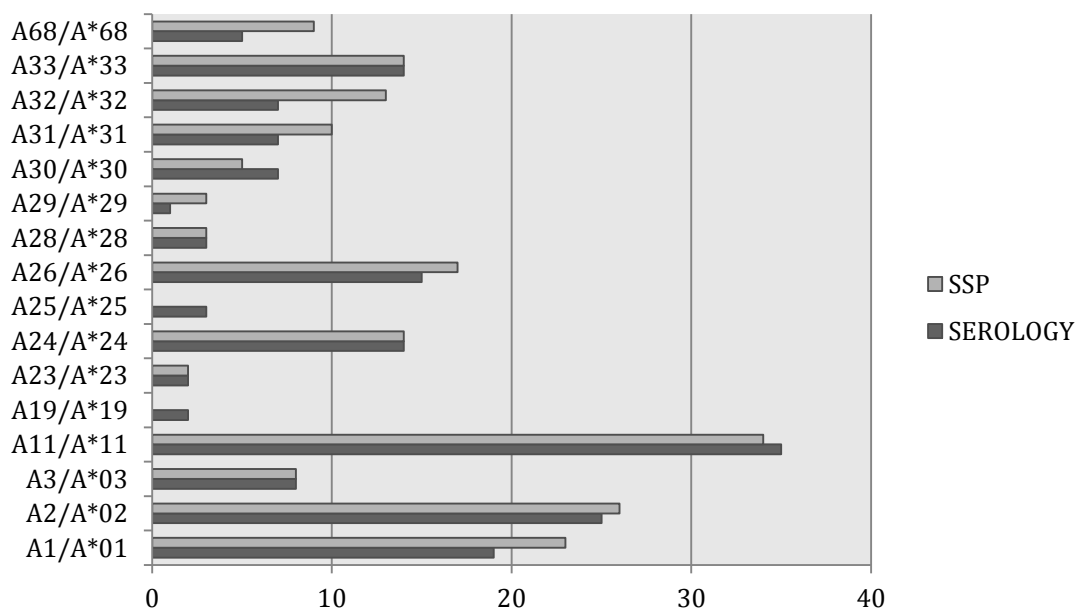


Figure 2. Antigens/alleles distribution for HLA A locus. X axis shows number of antigens detected against a particular antigen on Y axis.

SSP: sequence specific primers

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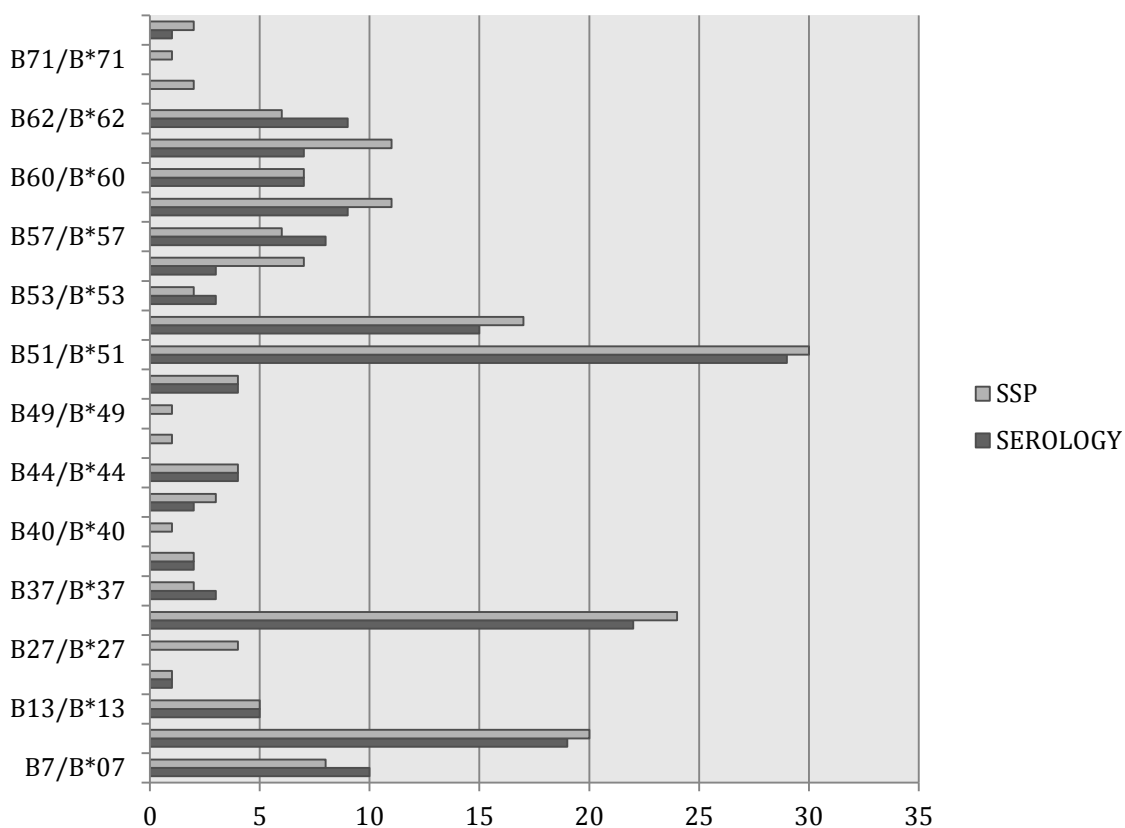


Figure 3. Antigens/alleles distribution for HLA B locus. X axis shows number of antigens detected against a particular antigen on Y axis

SSP: sequence specific primers

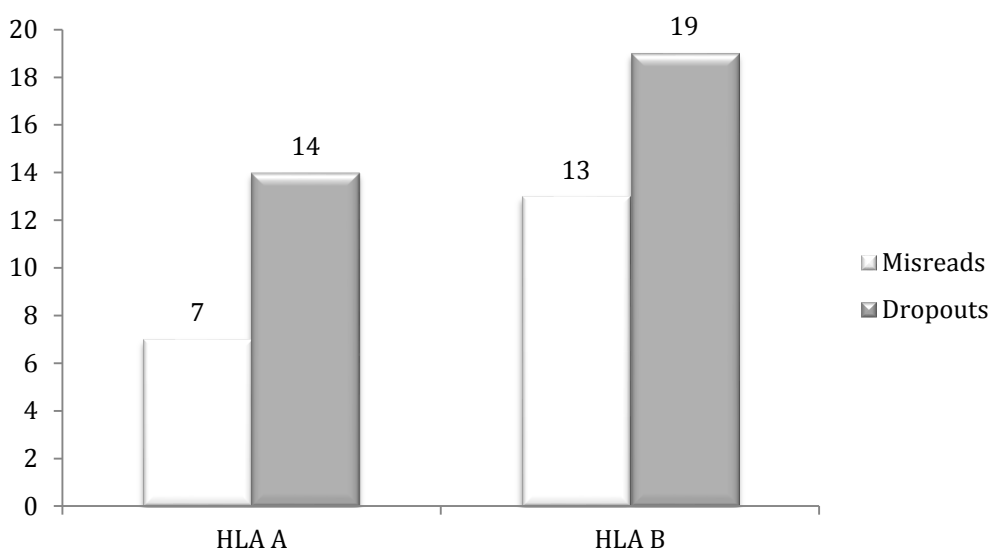


Figure 4. Total number of allelic misreads and dropouts using serological method, detected then by sequence specific primers (SSP)

Table 1. Distribution of allele misreads and dropouts for HLA A and B loci

HLA Locus (total alleles detected)	Allele	Misreads (antigens misread)	Dropouts	Total	Percentage (misreads + dropouts)
A (181)	A*01	-	4	4	11.6 %
	A*02	-	1	1	
	A*26	1 (A25)	-	1	
	A*29	1 (A19)	-	1	
	A*31	1 (A30)	1	2	
	A*32	2 (A11, A25)	4	6	
	A*68	-	4	4	
B (184)	B*08	-	1	1	17.4%
	B*27	1 (B37)	3	4	
	B*35	-	2	2	
	B*41	-	2	2	
	B*48	-	1	1	
	B*50	-	1	1	
	B*51	2 (B52)	-	2	
	B*52	3 (B51)	1	4	
	B*55	1 (B53)	3	4	
	B*58	2 (B57)	-	2	
	B*61	-	5	5	
	B*63	2 (B62)	-	2	
	B*71	1 (B41)	-	1	
	B*75	1 (B62)	-	1	

DISCUSSION

By comparing serology vs SSP, we detected 167 vs 181 and 165 vs 184 alleles for HLA A and B loci, respectively. SSP technique was clearly more sensitive in detection as Mishra et al⁹ also detected 8% and 20% more alleles for HLA A and B loci using SSP.

In this study, we were able to identify several antigens at both HLA A and B loci that are frequently either misread on serology or are missed altogether. Such antigens are more common on B locus and this is expected, because this locus is the most polymorphic one among all HLA loci.¹⁰ Allelic dropouts were more common than misreads. HLA A32 was the commonest HLA A antigen that was both misread and missed on serology while B52 and B61 were such antigens for HLA B locus. Prasad et al have found A2 and B35 to be disparate in 24% and 60% donor recipient pairs.¹¹ Both of these antigens showed disparity also in our results. Narouei-Nejad et al have found disparity of 31% between two methods, with SSP additionally

detecting A*24, A*28, B*18, B*16 and B*48.¹² We also found B*48 in one patient as not detectable on serology although no difference was found in other antigens. Similarly Shen et al have found 2.49% and 1.49% serological non-identification for HLA A and B loci respectively, when compared to sequence specific oligonucleotide probes (SSO).¹³ Discrepant results have also been noted even among different DNA-based techniques. Ribas-Silva et al have found HLA A*03, A*30, B*27, and B*45 alleles to be problematic when compared by SSP and SSO.¹⁴ We also had disparate results with A30 and B27.

Variations in identification of problematic antigens are expected, not only because of observer's variability, but also due to different antigen frequencies in various populations and different antisera/commercial plates used for serological identification. Most of the antigens that had been misread by us on serology were either splits of same broad specificities (A*26 and A25, A*29 and A19, A*31 and A30, B*51 and B52, B*52 and B51, B*58 and B57, B*63 and B62, B*75 and B62) or

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belonged to the same cross-reactive groups (CREGs) (A*32 and A11).¹⁵ This highlights the fact that one should be particularly careful while interpreting antigens identified in Table 1, especially the splits and cross reacting groups (CREGs). Haplotype matching within the family may help to some extent. Ambiguities in serologic-based method, if any, must be resolved with the help of DNA-based techniques.

Ambiguous results, including misreads and dropouts are not uncommon in HLA typing. Such findings may accrue from technical limitations as well as from inherent problem with serological methods. Many DNA-based methods are available to troubleshoot such eventualities. Family studies make a significant contribution to the resolution of difficult haplotypes.

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