Research Article

Challenges and Clinical Significance of Virtual Crossmatch in Kidney Transplantation: Our Experience

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Abstract

Background: The presence of anti-HLA antibodies, especially the presence of donor specific antibodies was associated with graft rejection after transplantation. The aim of our work was to analyze whether there is a correlation between actual-crossmatch performed by Luminex and virtual-crossmatch assessed on the basis of recipient's anti-HLA antibody specificities.

Material and Methods: Anti-HLA antibodies screening ± identification and crossmatch tests were performed before renal transplantation in 310 potential recipients, using Luminex technology. For all patients and donors, pretransplant HLA genotyping for A, B, and DRB1 loci were performed using molecular biology methods. To perform virtual crossmatch, the recipient's HLA-antibody specificities were compared against the donor HLA alleles.

Results: The anti-HLA antibodies screening was positive in 65 recipients (103 positive results): 15 patients (23%) presented anti-HLA class I antibodies, 12 patients (18.5%) had anti-HLA class II antibodies and in 38 subjects (58.5%) we discovered both types of antibodies. Using LSA assay, we could determine the antibody specificities only in 87 cases. Comparing the recipient's anti-HLA antibody specificities with donor's HLA antigens we found positive virtual-crossmatch in 81 cases. For 620 crossmatch results, the sensitivity, specificity, positive and negative predictive values were 87.6%, 97.8%, 85.5% and 98.1%, respectively.

Conclusion: virtual-crossmatch assessed on the basis of recipient's anti-HLA antibody specificities had a good correlation with actual-crossmatch performed by Luminex and thus, had a high sensitivity in predicting donor-recipient immunologic compatibility. Using the virtual crossmatch may improve graft allocation strategy for kidney recipients reducing the waiting time on the waiting list.

Introduction

The Human Leukocyte Antigens (HLA) are highly polymorphic molecules that can present "self" and "non-self" peptides to the immune system and thus are critical for induction of an immune response. Due to their high polymorphism and high expression rate, "non-self" or mismatched HLA are major targets for the immune system. Indeed, after blood transfusions, pregnancies or transplants, the immune system may produce antibodies against the mismatched HLA. If these circulating HLA antibodies are directed against HLA of a subsequently transplanted organ, they bind to their targets on the vascular endothelial cells of the allograft. The bound HLA antibodies will then activate the complement system as well as macrophages and neutrophils leading to severe endothelial cell damage and allograft dysfunction. This clinico-pathological entity induced by preformed donor-specific HLA antibodies is called antibody mediated or humoral rejection and is responsible for most early allograft losses. Therefore, detection of HLA-DSA prior to transplantation is an important step in the assessment of the patient's immunological risk and for exclusion of incompatible donors.

The presence of circulating Donor-Specific Anti-HLA Antibodies (DSA), which occurs mainly through blood transfusion, pregnancy and previous organ transplantation, is associated with a high risk for early acute rejection and allograft loss. This limits the access to kidney transplantation [1]. In 1969, Patel and Terasaki [2] demonstrated the efficacy of Complement-Dependent Lymphocytotoxic Crossmatch (CDC) in defining immunological risk. For decades, it has been used as a standard method to determine the DSA presence before transplantation. A positive crossmatch represented an absolute contraindication to renal transplantation. However, in recent years, it has become evident that cell-based crossmatch has important limitations related to sensitivity and specificity. At present, the solid-phase assays, including ELISA or multiple beads–based technology (Luminex) are the most frequently used. Development of micro-particles coated with single HLA-molecules

(Luminex Single-Antigen Beads assay – LSA) allows an accurate detection and characterization of preexisting anti-HLA antibodies and some consider the Luminex technology to be the new gold standard for anti-HLA antibodies screening and identification [3]. Thereby, the presence of DSA can be determined "virtually" (virtual crossmatch; v-XM) by comparing the recipient anti-HLA antibodies with the donor HLA-alleles. The virtual crossmatch decrease the workload in HLA-laboratories and facilitates the organ allocation even in sensitized recipients [4-6]. On the other hand, because of the high sensitivity of the LSA technique and because of different current principles that are used in different laboratories to assess the presence of cytotoxic antibodies, some concerns have been expressed related to the ability to predict "true positive" crossmatches in the clinical scenarios. Several studies investigated the predictive value of the virtual-XM and conflicting results have been achieved [7-13]. At the moment there are no consensus protocols available.

The aim of our work was to analyze whether there is a correlation between actual-crossmatch (a-XM) performed by Luminex and v-XM assessed on the basis of recipient's anti-HLA antibodies identified by LSA technique. We have investigated the correlation between performed DSA strength and crossmatch results.

Material and Methods

Between 2013 and 2015, a number of 310 potential recipients, 204 males (66%) and 106 females (34%), have been investigated. Anti-HLA antibodies screening ± identification and crossmatch tests were performed before renal transplantation using Luminex technology. One Lambda kits (a Thermo Fisher Scientific Brand) were used for cytotoxic antibodies assessment: LabScreen® PRA for antibodies screening, while LabScreen® Single Antigen Class I (HLA-A, -B, -C) and Class II (HLA-DRB, -DQB, -DPB), were used to identify the specificity of anti-HLA antibodies. Crossmatches were worked with LIFECODES Donor Specific Antibody (Immucor Transplant Diagnostics, Stamford, USA). Labscreen and LSA were considered positive when the Mean Fluorescence Intensity (MFI) was higher than 1500. Patients with previous sensitizing events were evaluated for the presence of anti-HLA antibodies in the current and historic sera.

For crossmatches, donor lymphocytes isolated from peripheral blood or spleen were used as the source material for HLA. DSA includes a single blend of Luminex beads. Two of the beads in the blend are conjugated with monoclonal antibodies specific for HLA Class I or HLA Class II. When mixed with a lysate, these two beads will capture the solubilized HLA, making a donor-specific HLA target for antibodies in a serum sample. To determine if a capture bead was positive or negative for donor specific antibodies, the MFI value of each capture bead was compared to three cutoff values (background adjustment factors; BAFs). The three cutoff values were calculated from the background measured on the three control (CON) beads in each test well. The cutoff value calculated for a CON bead was subtracted from the MFI value of the capture bead (Adjusted MFI Values). A sample was considered to be positive for donor specific antibodies if two or more Adjusted MFI Values were positive.

Pretransplantation, for all patients and donors, HLA genotyping for A, B, and DRB1 loci were performed using molecular biology methods: Polymerase Chain Reaction (PCR) with sequence-specific primers (All Set™ Gold HLA ABDR Low Res, Invitrogen, USA).

To perform v-XM, the recipient's HLA-antibody specificities were compared against the donor HLA alleles. A positive result was defined as the presence of at least one HLA-antibody directed against an HLA molecule of the donor. Because we routinely typed HLA-A, B, and DRB1, we did not consider anti-HLA-C and DQB1 in performing v-XM. When relevant, donors were typed for these loci, too. All investigations were done in Fundeni Clinical Institute, Centre for Immunogenetics.

Results

We have investigated 310 potential kidney recipients and 87 living/deceased donors regarding HLA compatibility, current cytotoxic antibodies and current crossmatches for class I and II. In 28 patients having a history of sensitizing events (10 patients with blood transfusion, 3 with previous renal transplantation and 15 multiparous women) we have also evaluated the presence of anti-HLA antibodies in the historic sera.

In the most cases (54%), the HLA-A, B, DRB1 compatibility between donor and recipient was 50% (3 mismatches), other 87 pairs (28%) had 4 mismatches and a smaller percent of pairs (10.3% and 7.7%) had 5 and respectively 2 mismatches. The anti-HLA antibodies screening was positive in 65 recipients (21%): 15 patients (23%) presented anti-HLA class I antibodies, 12 patients (18.5%) had anti-HLA class II antibodies and in 38 subjects (58.5%) we discovered both types of antibodies. The PRA (Panel Reactive Antibody) average was 29.73% (range 7-95%) in class I and 44.93% (range 8-98%) in class II. MFI value ranged between 1528 and 8730. Next step, in sensitized patients, was the cytotoxic antibodies identification. Using LSA assay for testing the 103 positive results obtained at screening, we could determine the antibody specificities only in 87 cases (47 class I and 40 class II), considering as positive result when MFI was ≥1500. In the most cases, we found a mixture of antibodies, anti-HLA-A+B+C (38.3%), anti-HLA-A+B (34%) or anti-HLA-B+C (4.3%) for class I specificities, and anti-HLA-DRB+DQB (30%), anti-HLA-DRB+DQB+DPB (27.5%), anti-HLA-DQB+DPB (7.5%) or anti-HLA-DRB+DPB (5%) for class II specificities. In the other cases, we found single specificities: anti-HLA-A (17%), anti-HLA-B (6.4%), anti-HLA-DRB (12.5%), anti-HLA-DQB (17.5%). We did not have any cases with anti-HLA-C antibodies alone (Table 1).

We compared the MFIs of the single-antigen beads with actual positive or negative crossmatch results for both class I and class II trying to determine to what extent an MFI cut-off could be used to predict crossmatch outcomes. Comparing the recipient's anti-HLA antibody specificities with donor's HLA antigens we found positive v-XM in 81 cases. The v-XM results predicted by LSA-DSA had a good correlation with a-XM performed by Luminex (Figure 1).

Table 1: Antibody specificities.

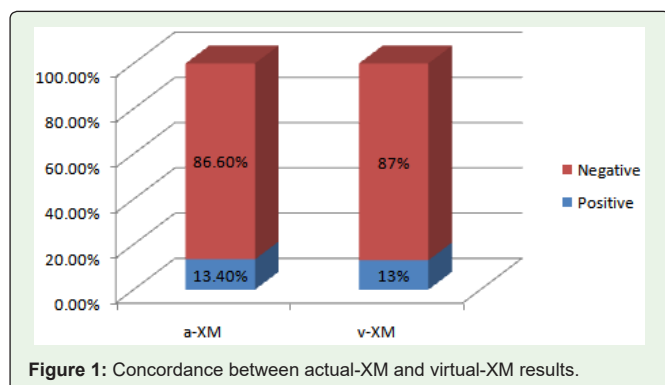| Class I specificities (anti-HLA)	N (%)	Class II specificities (anti-HLA)	N (%)
A+B+C	18 (38.3)	DRB+DQB+DPB	11 (27.5)
A+B	16 (34)	DRB+DQB	12 (30)
B+C	2 (4.3)	DQB+DPB	3 (7.5)
A	8 (17)	DRB+DPB	2 (5)
B	3 (6.4)	DQB	7 (17.5)
		DRB	5 (12.5)

Figure 1: Concordance between actual-XM and virtual-XM results.

Table 2: Comparison between actual-XM and virtual-XM results by DSA against donor-specific HLA antigens assessment.

		Virtual-XM	
		Positive	Negative
Actual-XM	Positive	71	12
	Negative	10	527

For 620 crossmatch results, the sensitivity, specificity, positive and negative predictive values were 87.6%, 97.8%, 85.5% and 98.1%, respectively (Table 2).

Ninety eight of the 310 patients, all with negative actual-XM for both class I and class II, had kidney transplantation and they were continuously followed. In the first six months, sixteen (16.3%) transplanted patients, had a transient increased serum creatinine levels. In the clinical context, based on the favorable response to methylprednisolone pulse therapy but in the absence of renal biopsy, these events were interpreted as acute rejection episodes. For nine of these recipients, although the actual-XM were negative with current sera, LSA assay revealed the presence of donor-specific anti-HLA antibodies in current sera in three patients and in historic sera in six evaluated patients.

Discussion

This study was designed to assess the accuracy of our v-XM protocol, based on LSA for DSA identification, in predicting the results of a-XM in kidney transplantation. The concept of the virtual-XM is not new, but its broad implementation has been hindered by the lack of sensitive assays to define the precise specificities of anti-HLA antibodies [14]. With the introduction of solid-phase assays– such as beads array technology– the clinical application of the virtual-XM became a reality. The use of v-XM allows to safely exclude a prospective crossmatch reducing cold ischemia time. Published studies have shown low rejection rates and good allograft survival rate in sensitized patients transplanted with a negative virtual-XM [15,16].

However, there are two issues that need to be discussed: (i) positive actual-XM in the presence of negative virtual-XM and (ii) negative actual-XM in the presence of positive virtual-XM. The virtual crossmatch could be false negative due to incomplete HLA typing of the donor. The recipient could have a donor-specific antibody against an HLA allele epitope that is not covered by the current antibody panel. Donor-specific non-HLA antibodies could be missed by the

virtual crossmatch due to its restriction to HLA antibodies. The HLA molecules might be denatured during the production process exposing "novel" epitopes that are not present on properly configured HLA molecules *in vivo*. The detected HLA-DSA might be directed against HLA molecules found on Luminex beads but not *in vivo*. Probably, these antibodies are not pathogenic and they are of limited clinical relevance.

The results of our study have indicated that some discrepancies between a-XM and v-XM were due to incomplete donors HLA-genotyping or incomplete evaluation of the recipient's antibodies profile. In our centre, at the time of this study, we routinely genotyped HLA-A, B, and DRB1 for both donors and recipients and for this reason we did not take into account anti-HLA-C, -DQB and -DPB1 in interpreting v-XM. Unfortunately, at that time, we had not the possibility for HLA-DPB1 genotyping. Taking into account that we identified anti-DPB specificities in 40% of the samples with positive anti-HLA class II antibodies, we have interpreted that, probably in some cases, the positive a-XM results in the presence of negative virtual-XM could be a consequence of these antibodies. In five from 12 living related pairs with positive a-XM and negative v-XM, we could extend HLA genotyping at C and DQB1 loci. Reassessing v-XM we found positive results. The clinical relevance of such antibodies has not yet been firmly established and early acute rejection episodes due to isolated HLA-C or -DP antibodies are unlikely to happen. The problem is that they might cause a positive crossmatch [17,18].

A positive v-XM with negative a-XM can be explained by overestimation of HLA-antibodies. Unfortunately, there is no consensus on MFI cutoff. In some studies LSA was considered positive when the MFI was >500 [15] while in the other study DSA were considered "unacceptable" when the MFI was >5000 [19]. In our centre, the LSA MFI cutoff was established at 1500. This may increase the PRA level and also, the number of the antibody specificities. The clinical relevance of low level DSA at the time of transplant has been investigated by multiple groups and fortunately, the presence of low levels DSA is unlikely to result in hyper acute rejection [20-23]. Because complement is involved in acute mediated rejection, an assay distinguishing complement-fixing from non-complement-fixing DSA with high sensitivity and specificity would be useful and could lead to a better understanding of the effects of these antibodies on graft outcome. The C1q assay seems to be an highly sensitive and specific test that could identify complement-fixing antibodies. These antibodies were demonstrated to be clinically relevant in preliminary studies [24,25]. Using both Luminex Single Antigen and Luminex-C1q assays together, we could provide more useful information.

Conclusion

Our virtual crossmatch based on high resolution HLA-typing techniques combined with bead array-based HLA-antibodies techniques show high sensitivity in predicting donor-recipient immunologic compatibility. Introducing virtual crossmatching in kidney transplantation is of great interest in our country as we have to deal with a lot of sensitized patients. The good outcome of kidney transplanted patients with low DSA levels and negative actual-crossmatch, highlights the importance of evaluating DSA strength in implementing v-XM. Our opinion is that renal transplantations in the presence of a positive virtual-XM with a negative actual-XM it is not a contraindication. The recipients require an enhanced and

personalized immunosuppression combined with careful follow-up. Post-transplant DSA monitoring would allow detection of variations in antibody levels and may provide information for more efficient immunosuppressive protocol.

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