Original Article

Donor-Specific Antibody Detection by Single-Antigen Bead Assay for Renal Transplantation: A 2-Year Experience from South India

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Abstract

Introduction: Recipient sensitization against donor human leukocyte antigens (HLA) plays a key role in transplant rejection, and this risk is best minimized by efficient pre transplant antibody detection. Determination of antibody specificity with the highest sensitivity and degree of resolution to the allelic antigen level is achieved by using single-antigen bead (SAB) assay. **Methods:** This study evaluated the correlation of Luminex cross match (LXM) with SAB assay for detection of donor-specific antibodies (DSA). A total of 2075 renal transplant patients were screened for the presence of DSA by LXM, complement-dependent cytotoxicity (CDC) cross match, and 125 patients for SAB from January 2018 to December 2019. **Results:** There was a male preponderance among recipients ($P < 0.0001$), and the most affected age group was 21–40 years. HLA typing was done in 550/2075 by DNA PCR-reverse sequence-specific oligonucleotide probes (SSOP) method. HLA DSA by LXM was detected in 16.3% of recipients (338/2075). Majority 180/338 (53.2%) of the patients were class II DSA positive, ($P < 0.0001$). Among the class II DSA positive patients, 20/180 (11.1%) samples gave false-positive results by LXM. SAB for class I and class II HLA IgG antibodies was done in 125/338 renal transplant recipients, which included 20 recipients with false-positive class II Luminex DSA, to check whether the DSA detected were really donor specific or not. The results showed that although 20/125 patients had some antibodies detected in their serum, they were not against the donor HLA antigens, as per the HLA typing reports of the donors. When compared to SAB assay, LXM showed more discrepant results, particularly to class II DSA. **Conclusion:** In conclusion, LXM, if used in combination with SAB assay and HLA typing of donors if necessary for virtual cross match, will help in avoiding unnecessary exclusion of donors for renal transplant recipients and also for post transplant monitoring of recipients, especially in cadaveric donor transplants.

Keywords: *Donor–specific antibodies, luminex cross match, renal transplantation, single antigen bead assay*

Introduction

Human leukocyte antigens (HLA) are a system of glycoproteins that help in presentation of peptides to the immune system.[1,2] HLA molecules, being highly polymorphic, often become the targets of antibody response, especially in people with history of transfusion, previous transplant, or pregnancy. Sensitization against donor HLA plays a key role in transplant rejection, and this risk is best minimized by efficient pretransplant antibody detection, effective pre-allocation crossmatching, and minimization of HLA mismatches between the donor and the recipient.[3] Although complement-dependent cytotoxicity (CDC) assay has been the gold standard for many years, this test is less sensitive and is also based on complement activation, which implies that antibodies such as IgG2 and IgG4 which do not fix complement are not detected. To overcome these problems, more sensitive assays such as flow cytometry crossmatch (FCXM) had been introduced, but still, they have a disadvantage that the target cell is a lymphocyte with many different (non-HLA) target molecules on its surface. Moreover, CDC-XM and FCXM require the availability of living cells, which is an inconvenience for their use in the monitoring of patients transplanted with a cadaveric donor.[4,5] FCXM is not widely done in India due to lack of uniformity in reporting and variation in cutoffs used to assign positivity.[6] To overcome these problems, HLA laboratories have introduced Luminex bead-based assays among their procedures

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to detect the presence of anti-HLA antibodies, because these techniques are more sensitive and specific and do not require the availability of living cells.

The Luminex anti-HLA antibody detection assay is a solid-phase assay in which purified HLA molecules, either in single or a combination of types, are attached to beads and these molecules will bind to anti-HLA antibodies in the recipient's serum.[7] The value of Luminex crossmatch (LXM) in actually predicting graft survival has been questioned.[8] In studies where it was compared to flow cytometry and single-antigen bead (SAB) assays, it showed more discrepant results, particularly to class II donor-specific antibody (DSA).[9,10] A failure to identify false-positive antibodies from the Luminex assay results in the associated HLA being listed as unacceptable mismatches and unnecessarily limits the patient's access to transplantation or results in the administration of unnecessary and costly treatments. This results in an increased risk of death on the waiting list, inequity for access to transplantation, and potential adverse effects of enhanced immunosuppression.[11,12] Determination of antibody specificity with the highest sensitivity and degree of resolution to the allelic antigen level is achieved by using SAB.[13-16] SAB results enable virtual crossmatching (VXM), when the crossmatch result can be predicted from the recipient's HLA antibodies if complete typing of the donor is available.[17]

In India, there is no standard testing protocol followed by all transplant centers. Some centers perform renal transplant based on only CDC-XM, but some perform other techniques of antibody detection and crossmatch, including flow and Luminex. The concept of SAB assay to detect DSA antibodies has recently been introduced in our center. There is no single assay perfect enough, so multiple assays shall be used to determine true antibodies.[18] With this background, this study was carried out with the main aim of correlating LXM results with SAB assay to determine the exact specificity of the HLA IgG class I and II antibodies in recipient serum against donor HLA antigens and to rule out false-positive class II DSA in patients coming for renal transplant at our center.

Materials and Methods

A total of 2075 renal transplant patients were screened for the presence of DSA by LXM and CDC-XM during the study period of 2 years from January 2018 to December 2019. Transplant was done only for patients who were DSA negative both by CDC-XM and LXM test. 1574 were live donors and 501 were cadaveric donors.

Renal transplant recipients are screened for the presence of DSA by CDC-XM and LXM. HLA typing of recipient and biologically related or unrelated donors is done before transplant at our center. History of sensitization events, including pregnancy, transfusion, or a previous transplant, is taken from each patient. Luminex SAB assay for class I and class II HLA was requested by the clinician under three conditions as follows:

a. to check whether the DSA detected were really donor specific or not for patients in whom the panel-reactive antibody was 30%–50% for selection of immunologically favorable donor;
b. if DSA was positive in sensitized patients, SAB was requested to monitor decrease in MFI values of DSA detected to plan the desensitization protocol pretransplant; and
c. posttransplant monitoring of patients suspected of antibody-mediated rejection.

CDC crossmatch

CDC crossmatch was performed for all 2075 renal transplant patients using the standard National Institute of Health (NIH) technique, and a score of ≥4 was considered as positive.

LXM for HLA class I and class II IgG antibodies by DSA test

HLA DSA detection was done for all 2075 renal transplant patients using Lifecodes DSA kit (Immucor GTI Diagnostics. Inc.Waukesha, Wisconsin, USA). Donor lymphocytes isolated from peripheral blood were used as a source material for HLA. To assure that HLA had been captured, a lysate control reagent (LCR) was run in parallel with the test. LXM was considered positive if the MFI was >1000 for HLA class I and class II IgG antibodies and negative if the MFI was <1000.

Modified LXM for HLA class I and class II IgG antibodies to rule out false positivity

For performing the procedure with native beads, incubation of the beads with donor lysate was omitted for 20 patients whose class II LXM was false positive. Other steps of the procedure for LXM remained the same.[19]

HLA typing

A total of 550/2075 recipient and donor pairs were tested for low-resolution HLA-A, B, and DRB1 typing by DNA PCR-reverse sequence-specific oligonucleotide probes (SSOP) on Luminex by Lifecodes HLA-SSO typing kit (Immucor Transplant Diagnostics. Inc, Waukesha, Wisconsin, USA) as per the manufacturer's instructions, and the results were interpreted by MATCH IT! DNA program on a Luminex platform**.** HLA typing for 501 cadaveric donors was done for VXM, if requested by the clinician for posttransplant monitoring of recipients. If HLA C, DQ, or DP antibodies were detected in the recipient's serum by SAB assay, donor HLA typing for C, DQ, and DP locus was done for VXM.

SAB assay class I and class II

Serum from patients was analyzed using Lifecodes Luminex Screening Assay class I and class II kit (Immucor,

Stamford, CT USA) to determine the exact specificity of the HLA antibodies. A total of 125/338 recipient serum samples which were positive by LXM were tested by SAB assay as per the manufacturer's instructions, and samples were analyzed using Xponent 3.1 software (Luminex Corporation, Austin, USA) for data acquisition. A serum sample was considered to be positive to a specific bead when the MFI raw value for this bead was >1500 in our study. The presence of SAB-DSA was determined by comparing the SAB HLA A/B/C/DR/DQB /DP antibody specificities on serological level with HLA A/B/C/DR/DQB/DP typing of the donor. If DQ and DP antibodies are detected by SAB assay in recipients' serum, then HLA typing for DQ and DP locus was done from the DNA of donor stored at −40°C for VXM

Statistical analysis

The sensitivity and specificity of LXM and SAB assay was calculated using Med Calc easy to use statistical software.

Results

A total of 2075 prospective renal transplant patients came for pretransplant testing in the years 2018 and 2019. Among the donors, 1437 were live related, 137 were live unrelated, and 501 were deceased donors. Among 1437 live related donors, there were 693 (48.2%) parents, 243 (16.9%) siblings, 442 (30.7%) spousal donors, and 59 (4.1%) children. Among the recipients, there were 1539 (74.2%) males and 536 (25.8%) females. Age distribution among the recipients was as follows: 209 (10%) were below the age of 20 years, 1269 (61.2%) were between 20 and 40 years, 520 (25%) were between 40 and 60 years, and 77 (3.7%) were above 60 years of age, as shown in Table 1.

All recipients were screened for presence of DSA by CDC-XM and LXM. LXM was positive in 338/2075 (16.3%) and negative in 1737/2075 (83.7%) renal allograft recipients. Out of 338 positive LXM, 104 (30.8%) were positive for class I DSA, 180 (53.2%) were positive for class II DSA, and 54 (16%) were positive for both class I and class II DSA, as shown in Table 2. Among 338 LXM positive cases, 40 (11.8%) recipients were posttransplant cases, 175 (51.7%) had history of blood transfusion, 103 (30.5%) had history of pregnancy, and 20 (5.9%) recipients did not have any history of sensitization, as shown in Table 3. CDC-XM was positive in 73/338 (6.8%) cases. No autoantibodies were detected in CDC-XM positive cases, and 54/73 (74%) were LXM positive for class I, 4/73 (5.5%) were class II positive, and 15/73 (20.5%) were both class I and class II positive.

SAB assay for class I and class II HLA IgG antibodies was done in 125/338 renal transplant recipients. Of them, 20 were male recipients with a history of only one unit of blood transfusion and had PRA between 30% and 50%. To check for immunologically favorable donors and class II DSA by LXM being suspected to be false positive, SAB assay was done to check and confirm whether the DSA detected were really donor specific or not. LXM without bead incubation of lysate was also positive among these 20 patients, indicating that positivity by LXM may be false positive. SAB class I and class II results showed that no antibodies were detected in these 20 recipients' serum against their donor HLA antigens, as per the HLA typing reports of the respective donors. Among the above 20 recipients two recipients had MFI values between 1500 and 3000, six had MFI 3000–5000, and 10 recipients had MFI >5000 for class II DSA by LXM, although SAB was negative among them. Five out of 10 recipients whose MFI was >5000 for class II DSA by LXM were also positive for CDC-XM, but VXM was negative by SAB, as shown in Table 4. Four/20 registered for cadaveric transplant had class II DSA MFI 3000–5000 by LXM, but VXM was negative by SAB, as shown in Table 5. Among 105 recipients, six (5.7%) were posttransplant cases, 53 (50.4%) had history of more than one unit of blood transfusion, and 46 (43.8%) had history of pregnancy. Among these 105 recipients, SAB class I was positive in 12 recipients (11.4%), SAB class II was positive in 40 recipients (38%), and SAB class I and II were positive in 53 recipients (50.5%). When the VXM was performed in all these 105 patients, the antibodies detected were directed against donor HLA antigens as per the HLA typing reports of the donors. Ten/501 (10%) cadaveric recipients were positive for VXM. Positive VXM results by SAB of 2/10 of the cadaveric recipients whose LXM DSA

Table 1: Age distribution of renal transplant recipients (*n*=2075)

Year	<20 years (%)	20-40 years (%)	40-60 years (%)	>60 years (%)	Total
2018	117 (5.6%)	651 (31.4%)	174 (8.4%)	9 (0.4%)	951
2019	92 (4.4%)	618 (29.8%)	346 (16.6%)	68 (3.3%)	1124
Total	209 (10%)	1269 (61.2%)	520 (25%)	77 (3.7%)	2075

Table 2: Total number of donor-specific antibody tests by Luminex done for renal transplant recipients (*n*=2075)

Year	Total no. of DSA tests	No. of tests negative (%)	No. of tests positive for class I	No. of tests positive for class II	No. of tests positive for class I and II	Total no. of positive DSA tests
2018	951	763 (80%)	58 (6.1%)	102 (11%)	28 (2.9%)	188 (20%)
2019	1124	974 (86.7%)	46 (4.1%)	78 (7%)	26 (2.3%)	150 (13.3%)
Total	2075	1737 (83.7%)	104 (5%)	180 (8.7%)	54 (2.6%)	338 (16.3%)

DSA=donor-specific antibody

MFI for class II was between 1500 and 3000 are shown in Table 6. If the SAB results showed detection of HLA C, DQ, and DP antibodies in recipient's serum, presence of DSA was confirmed by performing HLA C, DQ, and DP locus typing of the donors for VXM. The sensitivity and specificity of SAB was 100%, as shown in Table 7.

Discussion

Although the SAB assay has a very high sensitivity for antibody detection, CDC-XM still remains the most commonly used test in developing nations like India owing to its easy availability and financial restraints.[20] The pretransplant evaluation of DSA by only CDC-XM is likely to be associated with higher rejection rates and poor graft survival, especially in high-risk sensitized recipients.[21] Luminex SAB may be useful even in cost-limited settings due to better risk stratification before transplantation.[22,23] Both false-positive and -negative results can occur with any of the bead-based assays, making it imperative to interpret results in conjunction with clinical history.[24]

There was a male preponderance among recipients which is similar to earlier studies[25,26] in which the authors postulated that this may be due to differences in renal hemodynamics, the renin–angiotensin system, macrophage infiltration, and a protective role of estrogen. The most affected age group among recipients was between 21 and 40 years HLA DSA by Luminex was detected in 16.3% of recipients (338/2075), which is lower than those reported in studies from India where 25.4%[27] and 39.2%[4] were documented. Majority of the patients had class II DSA positivity, which is similar to that reported in previous studies from India[27,28] and abroad.[29] Among the positive class II DSAs, 20/180 (11.1%) samples gave false-positive results, which is higher than that reported in a recent study from India.[4] The native bead in the crossmatch format is

Table 3: History of sensitization among the renal transplant recipients positive for Luminex crossmatch (N=338)

Year	Posttransplant (%)	Blood transfusion (%)	Pregnancy (%)	No history (%)
2018	17 (5%)	115 (34%)	48 (14.2%)	8 (2.4%)
2019	23 (6.8%)	72 (21.3%)	43 (12.7%)	12 (3.5%)
Total	40 (11.8%)	175 (52.6%)	103 (30.5%)	20 (5.9%)

Table 4: Virtual XM results of 5/10 patients whose LXM class II DSA was positive with MFI values >5000

Case no.	Recipient	Donor	Class II DSA (MFI)	Donor HLA typing	SAB of recipient	Virtual XM	Suitable for transplant
1	Son	Mother	Positive (8435)	A*68:01, A*33:01 B*52:01, B*44:03 DRB1*01:01, DRB1*03:01	Class I- negative Class II- DRB1*05:01	No DSA detected Virtual XM negative	Yes
2	Husband	Wife	Positive (5421)	A*03:01, A*68:01 B*18:01, B*35:01 C: 06:02, C*03:02 DRB1*07:01, DRB1*14:04	Class I- A: 01:01, C: 01:02 Class II- DRB1*01:01, DRB1*09:01, DRB1*15:01, DRB1*10:01	No DSA detected Virtual XM negative	Yes
3	Father	Son	Positive (5850)	A*02:01, A*33:01 B*07:08, B*08:01 DRB1*10:01, DRB1*15:01	Class I- negative Class II- DRB1*11:01, DRB1*09:01, DRB1*15:01	No DSA detected Virtual XM negative	Yes
4	Husband	Wife	Positive (6500)	A*02:11, A*11:01 B*35:03, B*08:01 DRB1*03:01, DRB1*13:01, DQB1*02:01, DQB1*06:03	Class I- negative Class II- negative	No DSA detected Virtual XM negative	Yes
5	Cousin brother	Cousin brother	Positive (8520)	A*01:01, A*11:01 B*40:01, B*52:01 DRB1*15:01, DRB1*15:02, DQB1*03:02, DQB1*06:01 DPA1*03:01, DPA1*04:01	Class I- negative Class II- DRB1*01:01, DRB1*14:04, DPA1*02:01	No DSA detected Virtual XM negative	Yes

DSA=donor-specific antibody, HLA=human leukocyte antigens, LXM=Luminex crossmatch, MFI=mean fluorescence intensity, SAB=single antigen bead, virtual XM=virtual crossmatch

Table 5: Virtual XM results of 4/20 patients who underwent cadaver donor transplant with Luminex crossmatch class II DSA positivity with MFI values 3000-5000

Recipient	Donor	Class II DSA (MFI)	Donor HLA typing	SAB of recipient	Virtual crossmatch	Suitable for transplant
1	Cadaver	5000	A*02:11, A*01:01 B*40:06, B*57:01 DRB1*07:01, DRB1*15:01, DQA1*01:02, DQA1*01:03 DQB1*03:01, DQB1*06:01	Class I- negative Class II- DRB1*14:04, DQB1*04:01	No DSA detected in donor Virtual XM negative	Yes
2	Cadaver	3500	A*03:01, A*33:01 B*50:01, B*51:01 C*06:02, C*16:02 DRB1*07:01, DRB1*01:01, DQA1*01:02, DQA1*02:01 DQB1*02:02, DQB1*05:01	Class I- Negative Class II- DRB1*10:01, DQB1*06:01	No DSA detected Virtual XM negative	Yes
3	Cadaver	4605	A*01:01, A*02:11 B*40:06, B*57:01 C*06:01, C*07:02 DRB1*07:01, DRB1*15:01, DQA1*01:02, DQA1*01:03 DQB1*03:01, DQB1*06:01	Class I- negative Class II- DQA1*02:01 DQB1*04:01	No DSA detected Virtual XM negative	Yes
4	Cadaver	4800	A*02:11, A*68:01 B*40:01, B*15:05 C*03:03, C*12:03 DRB1*14:07, DRB1*14:04, DQA1*01:01, DQA1*01:01 DQB1*05:03, DQB1*05:03 DPA1*01:03, DPA1:01:03 DPB1*02:01, DPB1*04:01	Class I- negative Class II- DQA1*03:02 DPB1*01:01	No DSA detected Virtual XM negative	Yes

DSA=donor-specific antibody, HLA=human leukocyte antigens, MFI=mean fluorescence intensity, SAB=single antigen bead, virtual XM=virtual crossmatch

Table 6: Positive virtual XM results by SAB of 2/10 of the cadaveric recipients whose LXM DSA MFI for class II was between 1500 and 3000

Recipient	Donor	Class II DSA (MFI)	Donor HLA typing	SAB of recipient	Virtual XM
1	Cadaver	1600	A*30:01, A*26:01 B*52:01, B*57:01 DRB1*04:04, DRB1*07:01, DQA1*01:01, DQA1*01:03 DQB1*03:01, DQB1*05:03	Class I- Negative Class II- DRB1*07:01 DQB1*06:04	DSA detected against donor - DRB1*07:01 Virtual XM positive
2	Cadaver	2650	A*11:01, A*02:11 B*40:06, B*44:03 C*03:09, C*15:02 DRB1*07:01, DRB1*01:01, DQA1*01:01, DQA1*02:01 DQB1*03:03, DQB1*06:04 DPA1*01:03, DPA1*02:01 DPB1*14:01, DPB1*35:01	Class I- negative Class II- DRB1*01:01 DQB1*06:04	DSA detected against donor - DRB1*01:01, DQB1*06:04 Virtual XM positive

DSA=donor-specific antibody, HLA=human leukocyte antigens, LXM=Luminex crossmatch, MFI=mean fluorescence intensity, SAB=single antigen bead, virtual XM=virtual crossmatch

Table 7: Comparison of Luminex XM with SAB assay among renal transplant recipients (*n*=125)

Test	Positive (%)	Negative (%)	Sensitivity (95% CI)	Specificity (95% CI)	NPV (95% CI)	PPV (95% CI)
Luminex XM	125 (100%)	0 (0%)	100% (96.5-100)	50% (33.8-66.2)	100%	84% (79.4-87.7)
SAB assay	105 (84%)	20 (16%)	100% (96.5-100)	100% (83-100)	100%	100%

CI=confidence interval, Luminex XM=Luminex crossmatch, NPV=negative predictive value, PPV=positive predictive value, SAB=single antigen bead

coated with antibodies against a non-variable portion of the HLA antigen, enabling its attachment. Therefore, the interfering antibodies detected in 20 cases may be directed either against these antibodies or against the bead, as per earlier studies.[19] LXM test was repeated multiple times in some of these patients who were showing class II DSA positivity with the same donors before transplant. Among these 20 patients, 10 (50%) showed an MFI of >5000 for class II DSA, which may suggest it to be a significant risk factor for acute rejection as per an earlier study,[30] and this may lead to elimination of donor for the renal transplant recipient. In view of low sensitization history of receiving blood transfusion only one time in male patients and LXM being positive multiple times with the same related donors, the false positivity of class II DSA was suspected, and hence, further confirmation was done by performing modified LXM without bead incubation, and further confirmation for class I and II HLA antibodies was done by SAB assay among these 20 patients who had PRA between 30% to 50%. The results showed that although some antibodies were detected in the patient's serum, they were all not against the donor HLA antigens as per the HLA typing reports of the donors. Although HLA C, DQ, and DP typing was not included in pretransplant testing, if the results of SAB assay of recipients showed presence of HLA C, DQ, and DP, it was confirmed whether these antibodies were against donor or not by HLA C, DQ, and DP locus typing results of donor. The sensitivity of LXM was 100%, but specificity was only 50%, whereas the sensitivity and specificity of SAB assay was 100% in our study. The VXM results of 20 patients were negative, and hence, all these 20 patients underwent renal transplantation and all of them were followed once a week for the first 2 months, once in 15 days for the next 2 months, then once a month for the next 3 months, and after that, once in 2–3 months for posttransplant monitoring. No antibody-mediated rejection was detected among any of these patients during the study period of 2 years.

Among patients who had history of sensitization, 11/105 (10.5%) had MFI values of more than 10,000 by SAB assay. These patients were further managed by two or three rounds of plasmapheresis, intravenous immunoglobulins, rituximab, and DSA was repeated by SAB assay for monitoring decrease in the MFI values of antibodies detected against the donor before transplant. Identification of pretransplant DSA in living[31,32] and deceased donors[33] offers an opportunity to lower the antibody levels through desensitization and allows transplant. DSA characterization by SAB assays has enhanced donor selection, and our observation is like earlier studies.[34] LXM allows us to perform a real crossmatch using donor lysate, which is easier to store than living cells for post-transplantation studies. On the other hand, SAB assays allow us to take a VXM by predicting reactions against HLA specificities. Therefore, both SAB assay and LXM could be used to detect the presence of anti-HLA DSA in patient sera. Hence, positive crossmatch results should always be correlated to priming history, and their relevance should always be confirmed by multiple assays.

Conclusion

LXM along with CDC-XM, if used for screening, and SAB assay, when used for specificity of antibody determination against donor HLA antigens, will help in confirmation of whether false-positive DSA detected in non-sensitized recipients are really against the donor or not. From our study, we conclude that LXM, when used in combination with SAB assay in non-sensitized recipients and HLA typing of donors if necessary for VXM, will help in avoiding unnecessary exclusion of donors for renal transplant recipients and for posttransplant monitoring of recipients, especially in cadaveric donor transplants, where getting donor samples is not possible.

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The data generated in the study are from the results of routine laboratory testing.

Conflicts of interest

We confirm that all the authors have read and approved the manuscript and confirm that there are no conflicts of interest.

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