Comparison of Physical Crossmatch and Virtual Crossmatch to Identify Preexisting Donor-Specific Human Leukocyte Antigen (HLA) Antibodies and Outcome Following Kidney Transplantation

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Data Interpretation D
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Background: Physical crossmatch (PXM) and virtual crossmatch (VXM) are applied to identify preexisting donor-specific human leukocyte antigen (HLA) antibodies in patients awaiting kidney transplantation. Recently, high-resolution epitope analysis has emerged as a novel strategy for VXM. A retrospective clinical study compared PXM with VXM before kidney transplantation and recipient outcome following transplantation.

Material/Methods: Between August 2017 and March 2018, 239 patients underwent crossmatching and 94 patients received a donor kidney. A complement-dependent cytotoxicity (CDC) PXM assay and VXM using serological and epitope analysis identified donor-specific antibodies (DSA). Crossmatch results and clinical outcome at 3 months were compared.

Results: VXM identified serological DSA (sDSA), verified epitope DSA, and total epitope DSA in 74 (31.0%), 39 (16.3%), and 49 (20.5%) cases, respectively. Eleven cases (4.6%) had a positive PXM detected by the CDC assay. Of 94 kidney transplant recipients, 21 had preexisting sDSA but were negative in PXM; there was 1 case of delayed graft function (DGF) and no cases of hyperacute rejection or acute rejection. Of the rest of the 73 recipients who were negative for sDSA, 8 had acute rejection (*P*=0.253) and 19 had DGF (*P*=0.037). No significant differences were found in graft survival at 3 months.

Conclusions: High-resolution epitope analysis identified fewer cases with DSA compared with serological analysis. Because patients with and without sDSA had a similar short-term outcome in the setting of a negative PXM, the presence of preexisting sDSA, determined by VXM, should not be an absolute contraindication for kidney transplantation.

MeSH Keywords: **Cytotoxicity Tests, Immunologic • Epitopes • Histocompatibility Testing • Kidney Transplantation**

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Background

The preexisting anti-human leukocyte antigen (HLA) antibodies in candidates awaiting kidney transplantation have been considered the main obstacle to kidney transplantation. Since 1969, when Patel and Terasaki demonstrated an increased incidence of hyperacute rejection in recipients with positive complement-dependent cytotoxicity (CDC) results, CDC has been the gold standard method used for antibody detection and crossmatching for patients before kidney transplantation [1]. Although the CDC assay has been shown to lack sensitivity, it continues to be used worldwide, and a positive CDC result is regarded as a contraindication to kidney transplantation. However, even when enhanced by the use of antihuman immunoglobulin as a secondary antibody (AHG-CDC), the false-negative rate of this cell-based crossmatching method remains high, and sensitization to the HLA locus is unknown [2].

The application of solid-phase assays has revolutionized the way to detect antibodies before transplantation. The Luminex single-antigen bead (LSAB) assay is a quantifiable fluorescence test in which the purified single HLA is fixed to a given microparticle bead, and enables detecting a wide spectrum of specific antibodies with high accuracy and sensitivity [3]. The application of the LSAB assay has significantly improved the identification of sensitized transplant candidates and has begun to evaluate epitopes that may generate antibodies with varying patterns of association with rejection. Furthermore, when combined with HLA typing, the LSAB assay enables virtual crossmatch (VXM) and evaluation of calculated panel reactive antibodies (cPRA), which significantly improves the efficiency of organ allocation [4–6].

Antibodies that are detected in serum bind to the epitope, which consists of between 15–25 amino acid residues [7–9]. A functional cluster of 2–5 amino acids within a radius of 3.0–3.5 ångstrom (Å), or 0.1 nanometers (nm), in the epitope is called the eplet, which primarily determines the binding specificity [10]. Antigens that are classified in the same serological antigen group may have different eplets, leading to variability in immunoreactivity. Epitope-based matching has previously been highlighted in the prospective identification of low-risk mismatches for highly sensitized candidates, and its role in determining long-term outcome following kidney transplantation has been studied [11]. When evaluating preexisting antibodies, there can be inconsistent findings from VXM analyzed by serology or epitope, as well as between VXM and cell-based physical crossmatch (PXM). However, little is known about the relationship between these assays to evaluate preexisting antibodies, or their clinical significance.

Therefore, this retrospective study aimed to evaluate preexisting donor-specific antibodies (DSA) analyzed by serology and epitope identification and to compare these methods with cell-based PXM, and their clinical significance, including the short-term prognosis of recipients with or without DSA.

Material and Methods

Ethical approval and approval for organ donation

All the organs for kidney transplantation were from donation after citizen's death (DCD) or from close family members, as approved by the Ethics Committee of the Third Xiangya Hospital, Central South University, Changsha Hunan, Peoples' Republic of China. The study was approved by the Institutional Review Board of Third Xiangya Hospital, Central South University (No. 2018-S374).

Patients studied

From August 2017 to March 2018, the clinical records of 239 patients who were evaluated for kidney transplantation and who underwent transplant surgery at our center were retrospectively reviewed. All the cases had undergone a Luminex single-antigen bead (LSAB) assay for virtual crossmatch (VXM), and a cell-based complement-dependent cytotoxicity (CDC) assay with their donors. There were 94 patients with negative CDC results who received kidney transplantation, who were all from DCD or from close family members.

Human leukocyte antigen (HLA) typing

All the kidney donors and 198 of the 239 patients who were awaiting kidney transplantation were typed for Class I HLA genes (HLA-A, HLA-B and HLA-C) and Class II HLA genes (HLA-DRB1, HLA-DQA1 and HLA-DQB1) with sequence-specific oligonucleotide probes using the LIFECODES HLA SSO Typing Kit (Immucor, Inc., Norcross, GA, USA) in the laboratory of our center, which was accredited by the National Center for Clinical Laboratories, China. The remaining 41 cases, who had been on the transplant waiting list since before 2015, underwent serological typing for HLA-A, HLA-B, and HLA-DR. No data for HLA-DP typing were available.

Serum HLA antibody detection

Serum HLA antibody detection had been analyzed using current samples, or historical results for up to 12 months before kidney transplantation, using a Luminex-based commercial kit, the LIFECODES LSA Kit (Immucor, Inc., Norcross, GA, USA). The serum samples underwent no other procedures except for high-speed centrifugation and were not diluted before the Luminex single-antigen bead (LSAB) assay. The LSAB assay results were expressed as the mean fluorescence intensity (MFI)

and were analyzed by the MATCH IT!P® Antibody version 1.3 software (Immucor, Inc., Norcross, GA, USA). The cutoff value was set as the MFI of ≥1000 to determine a positive result, which was corrected for background fluorescence.

Virtual crossmatch (VXM)

Virtual crossmatch (VXM) analysis included serological and epitope identification. The serological analysis compared the serological HLA typing and antibody detection to identify the serological donor-specific antibody (sDSA). The highest MFI value was recorded if over one bead was positive for the same antigen. In cases of multiple DSA, both the total and the peak DSA MFI were recorded.

The epitope analysis was performed with HLAMatchmaker version 02.0 for HLA-ABC, and version 02.1 for HLA-DR, HLA-DQ, and HLA-DP (*https://www.epitopes.net*). HLAMatchmaker is a structurally based computer algorithm that can determine HLA matching at epitope level. This method was able to determine both verified and unverified eplets. The MFI of the immunized eplet was recorded. In cases of multiple DSA, both the total and the peak of the DSA MFI were recorded.

Physical crossmatch (PXM) with the complement-dependent cytotoxicity (CDC) assay

The complement-dependent cytotoxicity (CDC) assay was performed using fresh peripheral blood mononuclear cells (PBMCs) from the kidney donor. Briefly, 50 000 PBMCs were incubated with 10 μL of the patient serum and 10 μL of rabbit complement (Cat no. S7764) (Sigma-Aldrich, St. Louis, MO, USA) for 35 minutes at room temperature. Then, 5 μL of 7-aminoactinomycin D (7-AAD) (Cat no. 559925) (BD Biosciences, Franklin Lakes, NJ, USA) was added and incubated for another 10 minutes in the dark. The 7-AAD compound is a fluorescent intercalator that undergoes a fluorescence spectral shift upon association with DNA. The samples underwent fluorescence-activated cell sorting (FACS) using the BD FACSCanto II™ (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells that underwent complement-mediated cell death was corrected by the negative control. A positive result was regarded as >10% of dead cells.

Immunosuppression treatment following kidney transplantation

In this retrospective clinical review, most of the kidney recipients were found to have received induction immunosuppression therapy with the polyclonal rabbit anti-thymocyte globulin (rATG) Thymoglobulin®, 2–4 mg/kg for between 5–7 days (Genzyme, Cambridge, MA, USA), or the mouse anti-human monoclonal antibody to interleukin (IL)-2 receptor (CD25), basiliximab, (Simulect®) (Novartis, Basel, Switzerland) 20 mg

twice at day 0 and at day 4. The rATG was primarily used in sensitized patients with antibodies, in patients with repeated transplantation, and kidney transplants with a long cold ischemia time. All patients received similar triple maintenance immunosuppressive therapy, consisting of tacrolimus, mycophenolate mofetil, and steroids. No desensitization treatment or minimization of immunosuppression treatment were used in this study.

Clinical outcome

The clinical data from all the kidney transplant recipients were collected and included the data from the 3-month follow-up. Graft function was evaluated by measuring the estimated glomerular filtration rate (eGFR), calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (2009). Delayed graft function (DGF) was defined as the post-operative requirement for dialysis, or serum creatinine levels that increased or remained unchanged, or decreased by <10% per day during 3 consecutive days in the first week following kidney transplantation [12]. Acute rejection (AR) was diagnosed clinically and defined as acute kidney injury in the absence of other causes, with a response to high-dose steroids and/or rATG with a reduction in serum creatinine levels to baseline [13]. Graft failure was defined as a return to hemodialysis dependence or the requirement for further transplantation.

Statistical analysis

Continuous data were presented as the mean ± standard deviation (SD), and were compared using Student's t-test, Welch's t-test or the Mann-Whitney U test, where appropriate. Categorical data were compared using Pearson's chi-squared (χ^2) test or Fisher's exact test, where appropriate. The correlation between different methods was tested using Pearson's chi-squared (χ^2) test and was assessed by Cramér's V-value (Cramér's phi or ϕc), which measures the association between two variables. Kaplan-Meier probabilities of graft survival and recipient survival were compared using the log-rank test. Statistical analysis was performed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). A P-value of <0.05 was considered to be statistically significant.

Results

Human leukocyte antigen (HLA) antibody profiles

Of 239 patients who were awaiting kidney transplantation, 126 patients (52.7%) were sensitized with HLA antibodies, which were detected using the Luminex single-antigen bead (LSAB) assay. Among the sensitized patients, 32 patients (13.4%) had antibodies only to Class I HLA (HLA-A, HLA-B, and HLA-C),

Table 1. The results of virtual crossmatch (VXM) and physical crossmatch (PXM) in patients before kidney transplantation.

	Positive			Negative	Positive rate	P-value
	Class I	Class II	Both			
Virtual crossmatch						
sDSA	30	28	16	165	31.0%	
Verified eDSA	25	9	5	200	16.3%	<0.001
Total eDSA	26	13	10	190	20.5%	
Physical crossmatch						
CDC			11	228	4.6%	

VXM – virtual crossmatch; PXM – physical crossmatch; sDSA – serological donor-specific antibodies; eDSA – epitope donor-specific antibodies; CDC – complement-dependent cytotoxicity.

48 patients (20.1%) had antibodies only to Class II HLA (HLA-DRB1, HLA-DQA1, and HLA-DQB1), and 46 patients(19.2%) had antibodies to both classes.

Virtual crossmatch (VXM) and physical crossmatch (PXM)

The results of virtual crossmatch (VXM) and physical cross-match (PXM) were shown in Table 1. VXM included serological and epitope analysis, respectively. Serological donor-specific antibodies (sDSA) was present if the mean fluorescence intensity (MFI) of any bead bearing the serological HLA of the donor was ≥1000. There were 74 out of 239 patients (31.0%) that had sDSA, of which 30 patients only had sDSA to Class I HLA, 28 patients had only sDSA to Class II HLA, and 16 patients had both. The mean MFI of the total of the sDSA values was 9318±14749 (range, 1050–95 089), and the mean MFI of the peak sDSA was 5113±4829 (range, 1050–20 278).

The MFI cutoff value for epitope analysis with HLAMatchmaker was 1000. However, only 39 of 239 cases (16.3%) had epitope donor-specific antibodies (eDSA) for verified epitopes, of which 25 cases had only Class I eDSA, 9 cases had only Class II eDSA, and 5 cases had both. The mean MFI of the total verified eDSA was 11731±16683 (range, 1049–85 853), and the mean MFI of the peak verified eDSA was 6493±5143 (range, 1049–20 278). When accounting for all epitopes, including the unverified epitopes, eDSA were found in 49 cases (20.5%), of which, 26 cases had only Class I eDSA, 13 cases had only Class II eDSA, and 10 cases had both. The mean MFI of the total eDSA was 10 695±16 062 (range, 0–90 013), and the mean MFI of the peak eDSA was 5971±5230 (range, 0–20 278) for all epitopes.

PXM, which was performed with the modified CDC assay, detected only 11 (4.6%) positive cases. Of these patients, 10 cases had both sDSA and eDSA, and one case had neither sDSA nor eDSA. Comparison of positive rates of VXM with PXM showed a significant difference when evaluating the preexisting antibodies (*P*<0.001).

Correlation analysis

The relationship between the crossmatch methods was further evaluated using pairwise correlation analysis. When evaluating the preexisting antibodies, the results of VXM and PXM were significantly correlated for each pairwise comparison (*P*<0.001, Table 2). The Cramér's V-value showed that the results of the verified eDSA compared with total eDSA was the most significant association (Cramér's V=0.870). For sDSA compared with verified eDSA, the Cramér's V-value was 0.659. However, the PXM had lower Cramér's V-values compared with VXM, which was mainly due to the low positive rate of CDC assay.

Following a review of antibodies to each HLA locus, the results of sDSA, verified eDSA, and total eDSA differed. Comparison of sDSA with verified eDSA identified only 181 cases (75.7%) that were identical in all HLA *loci*, of which 165 cases were negative for antibodies. Of the 39 cases which were both positive for sDSA and verified eDSA, only 16 cases contained the same for each locus. The concordance of sDSA compared with total eDSA was slightly higher (189 out of 239 cases; 79.1%), which included 163 both negative cases and 26 out of 47 both positive cases. However, 2 cases became positive for total eDSA in the setting of negative sDSA, and both were positive for the eplet of 94IL in HLA-C*03: 02. The concordance was the highest for verified eDSA when compared with total eDSA (218 out of 239 cases; 91.2%), which was in accordance with the Cramér's V-value. In comparison, the concordance between PXM and VXM was much lower (Table 2).

Therefore, the low-resolution sDSA might no longer reflect the DSA in the high-resolution epitope analysis. According to the HLA locus and the result of epitope analysis, the MFI value of each sDSA was analyzed (Table 3, Figure 1). Overall, the sDSA which were positive in the epitope analysis had much higher MFI values when compared with the negative ones (6924.56 versus 2498.88; *P*<0.001). Similar results were found for Class I/II antibodies and each locus, except for HLA-A, although the

Table 2. Correlation analysis of virtual crossmatch (VXM) and physical crossmatch (PXM) in patients before kidney transplantation.

| | | sDSA | | Sum | P-value | Concordance | Cramér's V-value |
		Positive	Negative				
Verified eDSA	Positive	39	0	39			
	Negative	35	165	200	<0.001	75.7%	0.659
	Sum	74	165	239			
Total eDSA	Positive	47	2	49			
	Negative	27	163	190	<0.001	79.1%	0.713
	Sum	74	165	239			
CDC	Positive	10	1	11			
	Negative	64	164	228	<0.001	72.8%	0.285
	Sum	74	165	239			

| | | Verified eDSA | | Sum | P-value | Concordance | Cramér's V-value |
		Positive	Negative				
Total eDSA	Positive	39	10	49			
	Negative	0	190	190	<0.001	91.2%	0.870
	Sum	39	200	239			
CDC	Positive	9	2	11			
	Negative	30	198	228	<0.001	86.6%	0.389
	Sum	39	200	239			

| | | Total eDSA | | Sum | P-value | Concordance | Cramér's V-value |
		Positive	Negative				
CDC	Positive	9	2	11			
	Negative	40	188	228	<0.001	82.4%	0.334
	Sum	49	190	239			

VXM – virtual crossmatch; PXM – physical crossmatch; sDSA – serological donor-specific antibodies; eDSA – epitope donor-specific antibodies; CDC – complement-dependent cytotoxicity.

positive findings for the HLA-A locus had higher MFI values, it was not statistically significant (5344.22 versus 2687.95; P=0.088). Furthermore, the Class II sDSA were more likely to be negative in epitope analysis compared with Class I sDSA (negative rate, 67.7% compared with 39.1%; P=0.001). Therefore, the results of epitope analysis were associated with the MFI values and the HLA locus.

Outcome at 3 months following kidney transplantation

There were 94 patients with negative CDC results who received kidney transplantation. Of these recipients, 73 cases had no sDSA, while the other 21 cases had positive sDSA (total sDSA MFI, 1055–20 726; median MFI of total sDSA, 2075; peak sDSA MFI, 1055–11 995; median MFI of peak sDSA, 2035). The clinical characteristics and short-term prognosis at 3-month follow-up were shown in Table 4. The basic characteristics of the

patient groups, including age, gender, body mass index (BMI), the re-transplantation rate, dialysis time, donor source, number of HLA mismatches, and induction therapy showed no statistical difference. Eight cases had verified eDSA, all belonging to the sDSA positive group. None of the recipients had hyperacute rejection, even though pre-transplantation sDSA and verified eDSA were present in some patients.

The glomerular filtration rate (GFR) and the incidence of acute rejection (AR) between sDSA positive and the sDSA negative groups were not significantly different in the first 3 months posttransplantation. However, the incidence of delayed graft function (DGF) in the sDSA negative group was significantly higher compared with that of sDSA positive group (26.0% versus 4.8%; P=0.037), and the 8 patients with AR were all in the sDSA negative group (16.4% versus 0.0%; P=0.253), leading to the lower trend in GFR found in the sDSA negative group in the first 3 months.

Table 3. According to the human leucocyte antigen (HLA) locus and the result of epitope analysis, the mean fluorescence intensity (MFI) value of each serological donor-specific antibody (sDSA) was analyzed.

	Frequency	Rate	MFI Mean ±SD	P-value
Class I				
Positive	53	60.9%	6237.15±5166.62	<0.001
Negative	34	39.1%	2658.93±2421.30	
HLA-A				
Positive	27	71.1%	5344.22±4937.29	0.088
Negative	11	28.9%	2687.95±1865.18	
HLA-B				
Positive	18	56.3%	6992.83±4743.97	0.005
Negative	14	43.7%	3431.93±3216.74	
HLA-C				
Positive	8	47.1%	7550.50±6825.31	<0.001
Negative	9	52.9%	1421.00±499.56	
Class II				
Positive	20	32.3%	8746.20±5293.71	<0.001
Negative	42	67.7%	2369.31±2246.91	
HLA-DR				
Positive	5	26.3%	10829.40±5926.89	0.007
Negative	14	73.7%	2729.36±2662.58	
HLA-DQ				
Positive	15	34.9%	8051.80±5092.01	<0.001
Negative	28	65.1%	2189.29±2037.69	

HLA – human leukocyte antigen; MFI – mean fluorescence intensity; sDSA – serological donor-specific antibody.
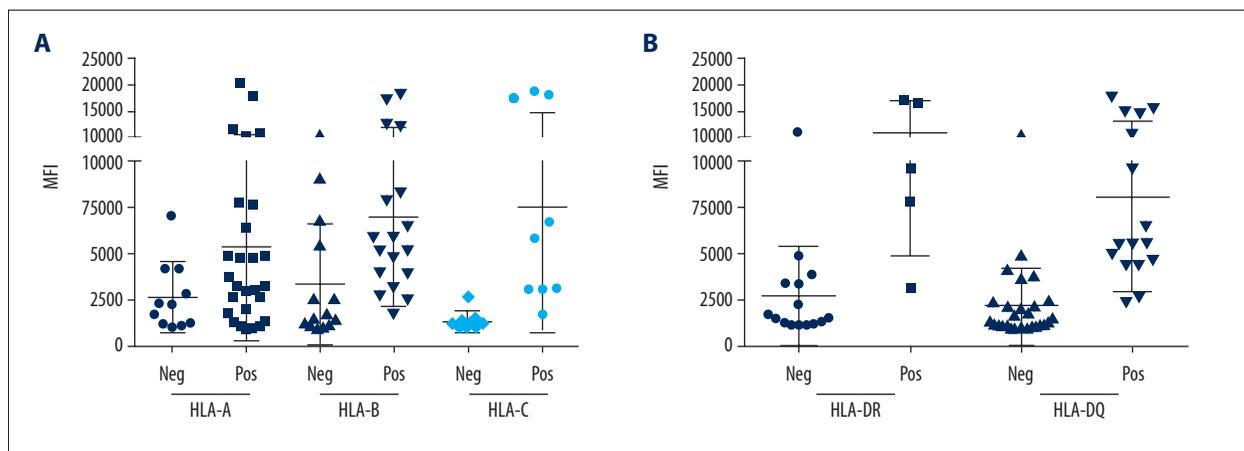

Figure 1. The results of human leukocyte antigen (HLA) epitope analysis using virtual crossmatch (VXM) with HLAMatchmaker show serological donor-specific antibodies (sDSAs) to each locus as shown by the mean fluorescence intensity (MFI). HLAMatchmaker is a computer algorithm that determines HLA matching at the epitope level. The results are classified epitope-positive and epitope-negative sDSA. (**A**) HLA-A, HLA-B, and HLA-C (Class I) sDSAs in the epitope analysis using VXM with HLAMatchmaker. (**B**) Class II sDSAs in the epitope analysis using VXM with HLAMatchmaker. DSAs – donor-specific antibodies; eDSAs – epitope donor-specific antibodies; HLA – human leukocyte antigen; MFI – mean fluorescence intensity; sDSA – serological donor-specific antibody; VXM – virtual crossmatch.

Table 4. The influence of the presence of serological donor-specific antibody (sDSA) on short-term outcome following kidney transplantation.

	All recipients (n=94)	sDSA-negative (n=73)	sDSA-positive (n=21)	P-value
Age (years)	41.22±10.74	40.84±9.95	42.57±13.32	0.517
No. of men (%)	63 (67.0%)	52 (71.2%)	11 (52.4%)	0.105
BMI (kg/m^2)	21.28±3.15	21.39±3.28	20.90±2.66	0.540#
Re-transplantation. No. (%)	2 (2.1%)	1 (1.4%)	1 (4.8%)	0.927*
Time since dialysis (months)	22.93±24.42	22.14±2.46	25.67±24.70	0.401#
Donor source				0.422*
DCD. No. (%)	83 (88.3%)	66 (90.4%)	17 (81.0%)	
Relative. No. (%)	11 (11.7%)	7 (9.6%)	4 (19.0%)	
HLA-A/B/DR mismatch	3.29±1.05	3.32±1.06	3.20±1.06	0.768#
Induction				0.829*
None. No. (%)	11 (11.7%)	8 (11.0%)	3 (14.3%)	
ATG. No. (%)	66 (70.2%)	51 (69.9%)	15 (71.4%)	
Basiliximab. No. (%)	17 (18.1%)	14 (19.2%)	3 (14.3%)	
Verified-eDSA positive. No. (%)	8 (8.5%)	0 (0.0%)	8 (38.1%)	<0.001*
Short-term clinical prognosis				
§GFR – mL/min/1.73 m^2				
0 week	4.65±1.81	4.58±1.67	4.88±2.26	0.506
1 week	47.14±33.52	43.50±32.82	59.63±33.67	0.052
2 weeks	51.81±29.49	49.87±28.93	58.62±31.18	0.244
4 weeks	57.88±21.34	55.61±21.16	65.58±20.66	0.066
12 weeks	70.39±22.16	69.13±20.85	74.70±26.32	0.338
Hyperacute rejection. No. (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	–
Acute rejection. No. (%)	8 (8.5%)	8 (11.0%)	0 (0.0%)	0.253*
Delayed graft function. No. (%)	20 (21.3%)	19 (26.0%)	1 (4.8%)	0.037*
Graft survival at 3 months. No. (%)	91 (96.7%)	70 (95.7%)	21 (100.0%)	0.351
Patient survival at 3 months. No. (%)	91 (96.7%)	70 (95.7%)	21 (100.0%)	0.35

* Tested by Fisher's exact test; # Tested by Mann-Whitney U test. § GFR calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (2009). ATG – anti-thymocyte globulin; BMI – body mass index; DCD – donation after citizen's death; eDSA – epitope donor-specific antibodies; GFR – glomerular filtration rate; HLA – human leukocyte antigen; sDSA – serological donor-specific antibodies.

For the short-term prognosis, 1 kidney transplantation recipient lost the graft due to acute cellular rejection on day 42 following transplantation. Another recipient lost the graft on day 11 following transplantation due to poor graft preservation (the histopathology of the kidney graft showed extensive necrosis).

Two kidney transplantation recipients, who had adequately functioning grafts, died on day 18 and day 31 following transplantation due to multiple severe infections. Another kidney transplant recipient underwent graft removal on day 16 due to infection from the donor, and the recipient died on day 31
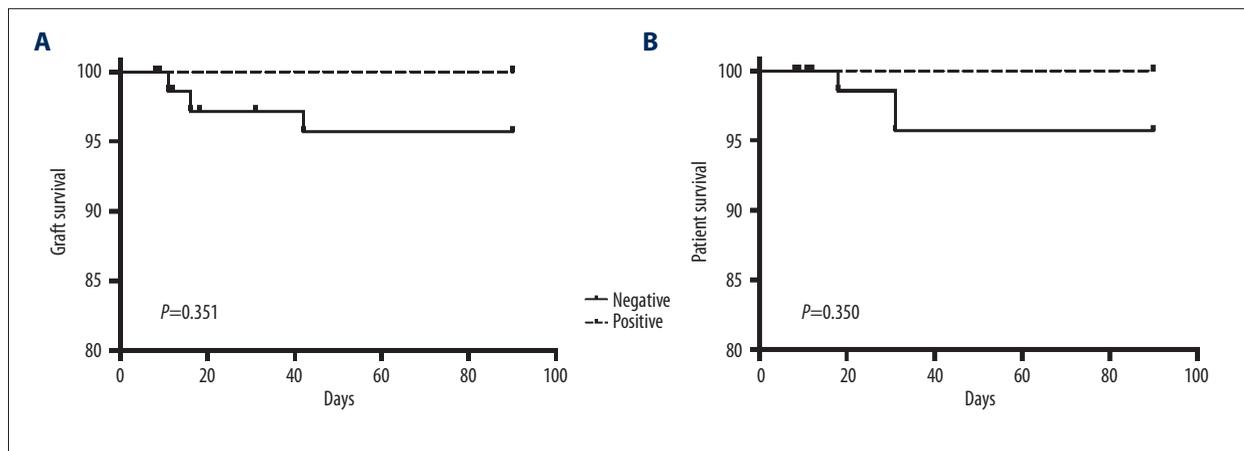
Figure 2. The short-term graft and patient survival of serological donor-specific antibody (sDSA)-positive and sDSA-negative groups after kidney transplantation There were 94 patients who underwent kidney transplantation with a 3-month postoperative follow-up. Before kidney transplantation, the mean fluorescence intensity (MFI) value of each serological donor-specific antibody (sDSA) was determined using the Luminex single-antigen bead (LSAB) assay. The pre-transplant sDSA-positive group (n=21) and sDSA-negative group (n=73) were compared. (**A**) Kaplan-Meier plot of death-censored graft survival at 3-month follow-up shows no significant difference between the sDSA-positive group and the sDSA-negative group (*P*=0.351). (**B**) Kaplan-Meier plot of patient survival at 3-month follow-up shows no significant difference between the sDSA-positive group and the sDSA-negative group (*P*=0.350). DSAs – donor-specific antibodies; LSAB – Luminex single-antigen bead; MFI – mean fluorescence intensity; sDSA – serological donor-specific antibody.

due to severe pneumonia. All these patients were from sDSA negative group. Death-censored graft survival and patient survival at 3-month follow-up showed no significant difference between the pre-transplant sDSA-positive group (n=21) and sDSA-negative group (n=73) (*P*=0.351 and *P*=0.350, respectively) (Figure 2).

Discussion

This study aimed to undertake a retrospective review to evaluate preexisting donor-specific antibodies (DSA) based on serology and epitope identification and to compare these methods with cell-based physical crossmatch (PXM) using a complement-dependent cytotoxicity (CDC) assay, and to evaluate patient outcome following transplantation. Preexisting alloantibodies against human leukocyte antigen (HLA) of the potential donor are recognized as the most important factor associated with hyperacute rejection and graft failure. New crossmatching techniques have been developed to evaluate alloantibodies present in sensitized patients, but the results vary between different methods. Therefore, the clinical relevance of serological donor-specific antibodies (sDSA) and epitope-specific antibodies (eDSA) was evaluated in this study.

When the result of the CDC assay is positive, it has been considered to be a contraindication to kidney transplantation [14]. However, the CDC assay lacks sensitivity and may lead to a false-positive result as well (1 case had no sDSA or eDSA, but

had a positive CDC result) [15]. Also, several factors make it technically challenging to perform and standardize [15]. In our study, the traditional CDC test was modified, replacing subjective cell counting and scoring using light microscopy and fluorescence microscopy by a more objective test using flow cytometry. The overall positive rate of the CDC assay was 4.6%, which was much lower than the VXM (31.0% for sDSA and 16.3% for verified eDSA). Because the majority of kidney donations in our center were from donation after citizen's death (DCD), and an immediate crossmatch result was required. The use of antihuman immunoglobulin enhanced CDC (AHG-CDC) or T-cell and B-cell cell sorting was not performed routinely, although increased sensitivity has previously been reported with these methods [15].

Compared with the CDC assay, the Luminex single-antigen bead (LSAB) assay has a much higher sensitivity and enables the detection of antibody specificity at the allele level. However, most transplant centers still utilize serological typing to identify DSA (sDSA). Unlike the donor cells used in the PXM, which contains the complete donor HLA repertoire, the LSAB assay usually contains only about 100 HLA alleles for each class, resulting in missing information for certain alleles, especially for common alleles that occur non-western populations, such as DRB1*08: 03 in the Chinese population, and the DQα/β alleles, which are both polymorphic [16,17]. The reactivity to these missing alleles could only be predicted by the selected ones within the commercial Luminex kit, which belong to the same serological antigen group [17]. Because of these limitations in

current serological crossmatching, the presence of sDSA may be overestimated.

However, VXM using epitope analysis has the advantage of information from high-resolution HLA typing and the LSAB assay and can determine the donor epitope-specific antibody (eDSA). One of the tools for epitope analysis is the HLAMatchmaker, which is an algorithm developed to predict the eDSA. HLAMatchmaker assumes that eplets, the small functional configurations of polymorphic residues of amino acids on HLA molecules, are potential immunogens that generate specific antibodies and that patients cannot produce antibodies against self eplets [9,18]. The repertoires of HLA-A, B, and C and HLA-DR, DQ, and DP eplets are based on the International Registry of HLA Epitopes (*http://www.epregistry.com.br*), which is continually updated. The HLAMatchmaker automatically identifies the mismatched eplets, and then determines the reactive ones by removing the negative ones according to the LSAB assay and the cutoff values. Both the antibody-verified and antibody-unverified eplets are included, based on determination of antigenicity with informative HLA antibodies.

In this study, eDSA had much lower positive rates than that of sDSA (verified eDSA 16.3%; total eDSA 20.5%; sDSA 31.0%) even with the same mean fluorescence intensity (MFI) cutoff value. Some patients with positive sDSA had negative eDSA, indicating that epitope analysis was more conservative in identifying the DSA than serological analysis. These findings are supported by clinical observations that the results of detection of preexisting antibody vary between different methods [19]. Clinically, this inconsistency could affect management decisions for patients on kidney transplant waiting lists. A clinical concern is that the LSAB may be too sensitive in detecting HLA antibodies, which could result in more patients being classified as sensitized candidates (126 out of 239 cases, or 52.7% in this study) and as a result, these patients may lose the opportunity for kidney transplantation. However, whether the antibodies detected by LSAB assay are clinically relevant remains controversial, especially in the setting of a negative PXM. Caillard et al. reported that the preexisting DSA was no longer present in 66% of the recipients after kidney transplantation, and only the persistent DSA was associated with an increased risk of kidney graft loss at 5 years when all the recipients were negative in the CDC assay [20]. Adebiyi et al. reported that when the flow cytometry crossmatch (FCXM) was negative, which was another method for PXM, the 1-year incidence of acute rejection and 5-year death-censored graft survival showed no significant difference between preexisting DSA positive and negative groups [13]. Also, the characteristics of the antibodies, including the avidity, complement-binding ability, and IgG subclass, and the class and conformation of the HLA, could all modulate the effect [21,22].

Recently, increasing numbers of literatures reported the development of single antigen bead assays in the management of kidney transplantation patients. Jucaud et al. reported the findings of a study on the use of LSAB assays and conformational variants of HLA-I on the beads, in an attempt to increase the concordance between this assay and FCXM results and to improve the prediction of antibody-mediated rejection (AMR) [23]. Conformational variants on iBeads, HLA-I beads, and heavy chain-10 (HC-10) beads were found, with a bead-specific MFI cutoff where the β2-free HC (β2fHC) or peptide-free-β2aHC (pepF-β2aHC) normalized donor-specific antibody levels were associated with the relevant anti-peptide-associated β2-microglobulin-associated HLA HC (pepA-β2aHC) reactivity that was associated with a positive FCXM [23]. Mathur et al. have recently reported their 3-year experience of the use of Luminex-based DSA crossmatching for kidney transplantation, and showed the assay to be more sensitive, specific, and cost-effective than CDC crossmatching before and after kidney transplantation [24]. Molina et al. reported that although the use of single-antigen bead (SAB) assays improved the success of transplantation, its high sensitivity might limit the allocation of kidney allografts for sensitized patients, and increase transplant waiting times [25]. The findings from the use of the SAB-C1q assay that allows the detection of C1q binding antibodies, showed that the unacceptable mismatch definition according to the SAB-C1q assay, could improve the risk stratification of rejection, leading to an increase in the currently limited allocation of kidney allografts and a shortened waiting time for highly sensitized patients [25].

Lefaucheur et al. recently reported the findings from an observational study that analyzed the association between preexisting HLA DSAs and the incidence of AMR and outcome in 402 cadaveric donor kidney transplant patients [26]. The 8-year graft survival was significantly reduced (61%) in recipients with preexisting sDSA compared with sensitized patients without sDSA (93%) and non-sensitized patients (84%) [26]. Peak sDSA MFI was a better predictor of AMR than baseline MFI (*P*=0.028), and transplant recipients with an MFI >6000 had >100-fold increased risk for AMR when compared with transplant recipients with an MFI <465 (relative risk, 113; 95% CI, 31–414) [26]. They suggested that the presence of HLA-DSA was not associated with patient survival, but the risk of both AMR and loss of the kidney graft was directly correlated with peak HLA-DSA levels [26]. The findings of this previous study also support that for sensitized kidney transplant recipients, quantification of HLA antibodies can be used to stratify immunologic risk, and guide selection of acceptable kidney grafts [26].

In this study, all the recipients were negative using the CDC assay, and the short-term prognosis of preexisting sDSA positive and negative groups was compared. None of the recipients suffered from hyperacute rejection, and graft function,

incidence of acute rejection (AR), graft survival, and recipient survival showed no significant difference, indicating sDSA were not detrimental to short-term prognosis. Only 8 of 21 sDSA positive recipients (38.1%) had verified eDSA. These findings suggested that when viewed at higher resolution, at the level of the epitope, the presence of sDSA might not be a contraindication to kidney transplantation.

This study had several limitations. Because this was a retrospective clinical study that relied on the information in patient records, the HLA typing information was not complete, lacking the information of HLA-DRB3/4/5 and HLA-DP, which might influence the accuracy of VXM for Class II HLA using HLAMatchmaker. Also, some recipients only had serological HLA typing information, and the number of sDSA and eDSA positive recipients was limited. In this study, only short-term prognosis at 3 months was retrospectively reviewed, and for the majority of the recipients, acute rejection was diagnosed clinically and was not confirmed by biopsy and histology.

Conclusions

The findings of this study showed that there were differences between virtual crossmatch (VXM) and physical crossmatch (PXM) in the detection and evaluation of preexisting antibodies in patients awaiting kidney transplantation. High-resolution epitope analysis was more conservative in identifying donor-specific antibodies (DSA) compared with low-resolution serological analysis. In the setting of a negative flow cytometry-based complement-dependent cytotoxicity (CDC) assay, recipients with serum antibodies to DSA (sDSA) had similar short-term prognosis compared with those without sDSA. The presence of preexisting DSA, determined by VXM, should not serve as an absolute contraindication for kidney transplantation.

Conflict of interest

None.

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