Association between flow cytometric crossmatching and graft survival in Thai cadaveric-donor kidney transplantation

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Summary

Background: **The flow cytometry cross-match (FCXM) technique is a sensitive method and has been reported to predict and protect graft rejection more efficiently than the conventional complement-dependent cytotoxicity cross-match (CDCXM) and the anti-human globulin-complement dependent cytotoxicity (AHG-CDC) methods.**

Methods: **We performed retrospective FCXM in 270 cadaveric donor kidney transplant patients with negative CDC and AHG. The correlation between FCXM with graft rejection and graft survival within 1 year to 3 years was analysed.**

Results: **There were 97 (35.9%) samples with positive FCXM. Only 7 (2.6%) of the 270 samples had evidence of antibody-mediated rejection (AMR) at the first year, which increased to 10 (3.7%) AMR samples after 3 years. Interestingly, there was a significant association between FCXM results with the graft outcome at 1 year (P = 0.046). However, when the association was analysed at 3 years after transplantation, it did not reach statistical significance. FCXM detected concordant positive results in 4 out of 8 samples. These samples had mean fluorescence intensity (MFI) of the donor-specific antibody (DSA) higher than 2,000. The DSA was identified by a single antigen bead.**

Conclusion: **Although positive FCXM, particularly for HLA class I, was significantly associated with graft loss from AMR within 1 year of transplantation in this study, there were a lot of FCXM false positives, as high as 35.9%. Additional studies are required to further assess the usefulness of FCXM in Thailand.** ***(Asian Pac J Allergy Immunol 2016;34:86-93)***

Keywords: *antibody-mediated rejection (AMR), cadaveric donor, flow cytometric cross-matching (FCXM), graft survival, kidney transplantation*

Introduction

Antibody-mediated rejection is one of the major complications of kidney transplantation that limit the long-term graft survival.[1] The detection of the preformed allo-reactive antibodies of the recipient to the donor kidney tissue is a mandatory process that could be achieved by one of several techniques, including the conventional method, complement-dependent cytotoxicity (CDC), flow cytometry cross-match (FCXM) and virtual cross-match.[2,3] The conventional method, CDC, and/or a modified method, anti-human globulin-complement dependent cytotoxicity (AHG-CDC), are routine pre-renal kidney transplantation laboratory work-ups in Thailand. The AHG-CDC method, developed by Johnson et al., provides higher sensitivity of antibody detection than the CDC method[4] However, these techniques have limitations due to the low viability of lymphocytes. On the other hand, FCXM neither requires cell viability nor depends on the complement fixation, but detects the binding of immunoglobulin molecules to target cells by

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immunofluorescence.[5] Therefore, low viability of lymphocytes is not a limiting factor for FCXM. Moreover, FCXM could detect not only the complement-fixing antibody but also non-complement fixing immunoglobulin. Therefore, FCXM is more sensitive than CDC and AHG-CDC. One previous report in the Thai population found that FCXM is 8-32 times and 4-16 times more sensitive than CDC and AHG-CDC, respectively. They also found 28.9% positive FCXM in patients with negative CDC.[6] In addition, several reports have shown that FXCM protects graft rejection more efficiently than CDC and AHG-CDC.[7-10] Many researchers have suggested that patients with negative results of both AHG-CDC and FCXM show a better graft survival than patients who are AHG-CDC negative but FCXM positive.[11-14] In contrast, other studies reported that FCXM might be too sensitive and did not correlate with graft rejection.[15-17] However, FCXM might be a good HLA cross-matching method in the Thai population but no clinical data of the correlation between FCXM and the conventional method of Thai patients are available so far.

In this study, we aimed to determine whether FCXM could be a standard cross-matching method for kidney transplantation in Thailand by studying the clinical correlation of FCXM to other methods in cadaveric donor kidney transplantation in Thailand.

Methods

Patients

We performed retrospective FCXM in 270 kidney transplant patients receiving cadaveric donor at the HLA laboratory, National Blood Centre, Thai Red Cross Society from 2006 to 2011. All of these patients had negative HLA cross-matching by CDC and AHG-CDC. The clinical parameter of the patients was obtained from the Thai Transplantation Registry.[18] The diagnosis of antibody-mediated rejection (AMR) was based on Banff criteria 2007, which were used at the time of the study recruitment.[19] AMR in this study required 3 criteria. First, evidence of antibody-mediated injury e.g., C4d staining in the peritubular capillaries was demonstrated. Second, evidence of morphological change was demonstrated at the microscopic level e.g., peritubular capillaritis, ATN-like tubular degenerative changes, fibrin thrombosis, necrotising or transmural arteritis. Third, serological evidence of circulating donor-specific antibodies was demonstrated. All 3 criteria are required to make a definite diagnosis of AMR, while 2 of the 3 criteria are regarded as suspicious for AMR.[20] In this study, we included both patients with definite AMR and suspicious AMR in the analysis. The patients with T cell-mediated rejection according to the Banff criteria 2007 without AMR were excluded from the study. Graft survival outcome was followed-up for at least 3 years. This study was approved by the Institution Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, IRB No.420/54

HLA tying

Recipients and Donors were analysed for HLA-A, -B, and -DR types by Micro-SSP™ (One lambda, Canoga Park, CA, USA) and LABType® SSO (One Lambda, Canoga Park, CA, USA). The mismatch of HLA between recipient and donor was calculated by the allocation criteria program of the Organ Donation Centre, Thai Red Cross Society.[21]

Screening of anti-HLA antibodies

Sera of the patients were obtained from each transplant candidate at monthly intervals. Anti-HLA antibodies was screened using the LABScreen Mixed kit (One Lambda, Canoga Park, CA, USA) with simultaneous HLA class I and class II antibodies detection by micro-beads coated with purified class I and class II HLA antigens. The frequency of panel reactive antibodies (PRA) and their HLA class I and/or class II specificity was determined by goat anti-human IgG coupled with phycoerythrin (One Lambda, Canoga Park, CA, USA). The fluorescence of each bead was recorded as the mean fluorescence intensity (MFI). An MFI more than 1,000 was considered positive. The percent of PRA greater than 50% received a score of 2 points and PRA greater than 80% received a score of 4 points according to the allocation criteria of kidney transplantation in Thailand.[21]

HLA cross-matching

The CDC was performed with T- and B-lymphocytes following the American Society for Histocompatibility and Immunogenetics (ASHI) guidelines.[22] T and B cells were separated from peripheral blood using the nylon wool column technique. Cells were incubated with serum for 60 minutes and then the complement was added. After adding the complement, cells were incubated for 60 minutes and then stained with eosin dye to determine cell viability. A positive cross-match was defined as any degree of cytotoxicity above 20%, which was not affected by dithiothoreitol (DTT).

The AHG-CDC is a test modified from CDC by adding AHG to enhance the sensitivity of this technique. After incubating the cell with serum for 45 minutes, the 3 washing steps with medium were done to remove excess antibody. The AHG was added to enhance the sensitivity and followed by complement. The reaction was read by inverted phase contrast microscopy. If there were dead cells at a level of greater than 20%, this was considered a positive result.

The FCXM technique used anti-human Immunoglobulin (anti-human IgG) antibody, labelled with fluorescence to detect antigen-antibody complex in the reaction based on the method described previously.[23] Briefly, the frozen lymphocytes from a cadaveric donor were kept in a liquid nitrogen tank and used in the experiment. Only samples with >50% viability of lymphocytes were included.

Pronase treatment was performed for B cell cross-matching by adapting a previous report.[24] Briefly, a total of 2-5 x 10^6 lymphocytes were incubated at 37°C for 15 minutes with 1 mL of pronase (Sigma Chemical, St. Louis, MO). The cells were then washed twice with cold phosphate-buffered saline.

Peripheral blood mononuclear cells (PBMC) at a concentration of $3x10^5$ cells were incubated with 30µl of patient serum for 30 minutes at 4°C. The negative control serum was derived from male donors with blood type AB, which was shown to be negative for anti-HLA antibody by CDC and solid phase assay (SPA) screening. The positive control serum was derived from pooled positive serum from sensitised patients with high PRA. Upon incubation with patient or control serum, cells were washed and stained with 1:50 FITC-conjugated AffiniPure F(ab')2 fragment goat anti-human IgG (Jackson Labs., PA, USA; cat.# 109-016-098), and subsequently with anti-human CD19-PE-labelled antibody (Beckman Coulter, CA, USA), anti-human CD3-PC5-labelled antibody (Beckman Coulter, CA, USA), anti-human CD20-PC7-labelled antibody (Beckman Coulter, CA, USA), and anti-human CD45-ECD-labelled antibody (Beckman Coulter, CA, USA) with a titre of 1:2.5 for each. Flow cytometric analysis was performed using a FC500 Beckman Coulter (Beckman Coulter, CA, USA). We determined the median channel shift fluorescence intensity relative to negative control (MCF). An MCF less than 50 was defined as negative. The cut-off values were established by cross-matching the negative control sera with PBMC obtained from healthy donors and calculating the mean channel fluorescence ± 2SD.

Donor specific antibody (DSA) identification

Due to the high cost of DSA, this was only measured in some patients to support the diagnosis of antibody-mediated rejection. DSA was identified by FlowPRA single antigen (One Lambda, Canoga Park, CA) in 10 patients. The positive DSA was defined as mean fluorescence intensity (MFI). MFI greater than 500 after the removal of background was defined as HLA-DSA positive.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 for Windows.[25] The comparisons between groups were analysed by Two-tailed Mann-Whitney test, correlation analyses by Spearman test and Two-tailed Fisher's Exact test was applied to assess the relative risk of graft rejection in patients with positive and negative flow cross-match. We compared graft survival over time between the two groups of patients with positive and negative cross-match by the Kaplan-Meier method. A value of $p < 0.05$ was considered statistically significant.[8,17]

Result

Demographics and graft rejection

Baseline characteristics and incidence of graft rejection were shown in Table 1. We found no significant association of any baseline characteristic with the graft rejection. Ten of the 270 patients (3.7%) showed AMR. Seven of 10 were male in both the recipient and donor groups. The median recipient age in the rejection group was 49 years (range 15-65) and in the non-rejection group was 46 years (range 10-74). The mean $\pm$ SD of HLA mismatch was 2.5 $\pm$ 1.3 and 3.3 $\pm$ 1.3 in the non-rejection and rejection groups, respectively. The PRA level, cold ischemic time, immunosuppressive drug and type of the induction therapy also did not show any clinical significance with graft rejection. The mean $\pm$ SD of cold ischemic time were 20 $\pm$ 2.5 and 21 $\pm$ 4.6 in the non-rejection and rejection groups, respectively.

Flow cytometric cross-matching and graft rejection

From the total of 270 samples with negative CDC and AHG, there were only 7 (2.6%) samples with evidence of AMR at the first year and a total of 10 (3.7%) AMR samples at 3 years. On the other hand, there were 97 (35.9%) samples that were positive for FCXM. The association of FCXM

Table 1. Baseline characteristic in kidney recipient with graft outcome

		Graft outcome		
		Non AMR	AMR	
	Total	(N=260)	(N=10)	*P*-values
Recipient	270			
Gender -male	162	155	7	0.379
-female	108	105	3	
Median age (range)		46 (10-74)	49 (15-65)	0.653
Donor	270			
Gender - male	209	202	7	0.401
- female	61	58	3	
Median age (range)		35 (2-67)	30 (16-58)	0.946
HLA mismatch (ABDR)				0.145
MM 0	17	17	0	
MM 1	39	39	0	
MM 2	80	75	5	
MM 3	64	64	0	
MM 4	51	48	3	
MM 5	18	16	2	
MM 6	1	1	0	
Mean ± SD		2.5 ± 1.3	3.3 ± 1.3	
Median		2	3	
Value of PRA				0.294
<5%	171	166	5	
>5%	78	73	5	
Cold Ischemic Time (hrs) Mean ± SD (range)		20±2.5 (1-36)	21±4.6 (14-27)	0.781
Immunosuppression				
Cellcept^{R}	96	92	4	0.257
Immuran^{R}	250	243	7	0.116
Neoral^{R}	133	128	5	0.748
Prednisolone	10	9	1	0.265
Prograft^{R}	163	148	5	1
Rapamune^{R}	258	250	8	1
Myfortic^{R}	210	204	6	1
Certican^{R}	264	256	8	1
Type of the Induction therapy				0.165
ATG*	9	9	0	
ALG**	1	1	0	
Simulect^{R}	52	49	3	
Zenapex^{R}	105	99	6	
Compath-1H^{R}	2	2	0	
Other	7	6	1	
No induction	94	94	0	

*Anti-thymocyte globulin, ** anti-lymphocyte globulin

Table 2. Flow cytometric cross matching result with graft outcome at 1 year after transplantation

	FCXM result	Total (%)	Graft outcome Non AMR (%)	Graft outcome AMR (%)
FCXM – ve	T-/B-	173 (64.1%)	170 (64.6%)	3 (42.9%)
FCXM +ve	T+/B+	28 (10.4%)	25 (9.5%)	3 (42.9%)
	T+/B-	33 (12.2%)	32 (12.2%)	1 (14.2%)
	T-/B+	36 (13.3%)	36 (13.7%)	0 (0%)
	Total	270	263	7
	Sensitivity		57%	
	Specificity		64%	
	Positive Predictive Value (PPV)		4%	
	Negative Predictive Value (NPV)		98%	

results with graft outcome at 1 and 3 years was shown in Tables 2 and 3, respectively. Interestingly, there was a significant association between FCXM results with the graft outcome at 1 year (Fisher's Exact test = 6.243, P = 0.046). Four out of the 7 samples (57.1%) with AMR were in the positive T cell FCXM group (T+/B+ and T+/B- FCXM results). The other 3 samples (42.9%) with an incidence of AMR were negative for FCXM (T-/B- FCXM result). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of FCXM and graft outcome at 1 year after transplantation was 57%, 64%, 4% and 98%, respectively, as shown in Table 2.

However, when the association was analysed 3 years after the transplantation, it did not reach statistical significance (Fisher's Exact test = 4.255, P = 0.149). The Kaplan-Meier graft survival plot was shown in Figure 1. The donor-specific antibody (DSA) was only found in 10 patients with the suspicion of AMR due to the high cost of the test (Table 4). We found 8 samples, which have HLA antibodies specific to the donor antigen (patient No. 1-5, 8, 9, and 10), as shown in Table 4. The DSA

Table 3. Flow cytometric cross matching result with graft outcome at 3 year after transplantation

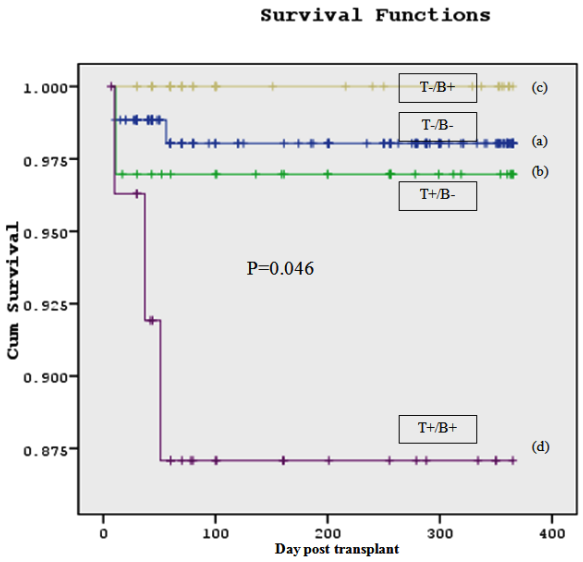
FCXM result	Total (%)	Graft outcome	
		Non AMR (%)	AMR (%)
T-/B-	173 (64.1%)	167 (64.2%)	6 (60%)
T+/B+	28 (10.4%)	25 (9.6%)	3 (30%)
T+/B-	33 (12.2%)	32 (12.3%)	1 (10%)
T-/B+	36 (13.3%)	36 (13.9%)	0 (0%)
Total	270	260	10

type allo-antibody results with a reciprocal value of median fluorescence intensity (MFI) were demonstrated by DSA (MFI) as follows: A24 (4,895), A2 (2,277), DQ8 (1,067), DR4 (611.6), DR53 (958), A203 (976) with DRB1*0402 (558), A11 (4,560) and B76 CREG B62 (5,322). It should be noted that some DSA could not be interpreted with certainty since we did not have full and high resolution HLA typing. Interestingly, FCXM can detect concordant positive results with DSA in only 4 of the 8 samples with an MFI value higher than 2,000 (patient No.1, 2, 9, and 10). In the other 6 patients with a lower MFI (patient No. 3, 4, 5, 6, 7, and 8), FCXM was negative.

Discussion

Many laboratories have set up and perform FCXM in addition to the CDC method for kidney transplantation.[16,23] This method is clearly more sensitive than the CDC method. Our results, in the comparison between percentages of FCXM positive with CDC cross-match (CDCXM) negative, were reciprocal to other studies. In our study, we found 97 (35.9%) positive FCXM out of 270 negative CDCXM patients. Kokthatong et al. found 21 (54%) positive FCXM out of 39 negative CDCXM[6] and Bishay et al. found 194 (54%) positive FCXM out of 357 negative CDCXM,[26] respectively. This demonstrated a high rate of false positive results for FCXM compared with clinical AMR, as shown in Tables 1 and 2. Nevertheless, some studies showed the benefit of positive T cell and B cell FXCM to determine graft outcome, particularly in the highly-sensitised group.[7,8,12,27,28] However, recent reports suggest that FCXM has less of a benefit in the low

1A.



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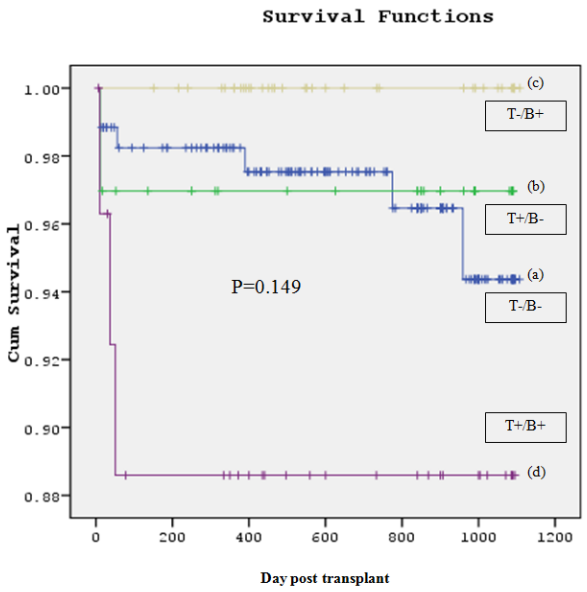

Figure 1. Kaplan-Meier graft survival by flow cytometrycrossmatch (FCXM) result
1A) One year survival curve, 1B) Three year survival curve

(a) patients that have T and B cell FCXM negative result [T-/B-]
(b) patients that have T cell FCXM positive result [T+/B-]
(c) patients that have B cell FCXM positive result [T-/B+]
(d) patients that have T and B cell FCXM positive result [T+/B+]

Table 4. DSA identification of 10 patients that have incidence of graft loss from AMR

Patient No.	Serum No.	Days to AMR Onset	% PRA	HLA Typing		DSA*		FCXM*	
				Recipient	Donor	DSA class I (MFI)	DSA class II (MFI)	T cell (MCF)	B cell (MCF)
1	50S0166	11	41	A2, A203; B13, B46; Bw4, Bw6; DR12, DR16; DQ5, DQ7	A2, A24; B13, B35; Bw4, Bw6; DR12, DR16; DQ5, -	**A24 (4894.9)**	DR52 (1814)	**Positive (95)**	Negative (26.9)
2	52G0589	37	83	A2, A11.1; B35, B46; Bw6; DR4, DR15; DQ4, DQ6	A2, A29; B44, B56; Bw4, Bw6; DR1, DR4; DQ5, DQ8	**A2 (2277)**	Negative	**Positive (76)**	**Positive (120.3)**
3	49P0931	390	0	A1, A33; B35, B58; Bw4, Bw6; DR13, DR15; DQ5, DQ6	A2, A24; B61, B62; DR4, DR15; DQ6, DQ8	Negative	**DQ8 (1067)**	Negative (-48)	Negative (-90.8)
4	49C0630	1276	0	A2, A11.1; B39, B61; Bw6; DR13, DR14; DQ5, DQ7	A26, A33; B39, B58; Bw4, Bw6 DR4, DR13; DQ6, DQ8	Negative	**DR4 (611.6)**	Negative (-75.9)	Negative (-52.4)
5	52G1301	56	65	A203, A11.1; B13, B62; DR15, -; DQ5, DQ6	A24, A33; B13, B44; DR7, DR15 (DR51, DR53)	A24 (476)	**DR53 (958)**	Negative (22.1)	Negative (-33)
6	53P0931	7	0	A11.1, -; B56, B61; Bw6; DR15, -; DQ5, DQ6	A2, A11; B13, B56; DR15, -; DQ5, DQ6	Negative	DQ6 (332.28)	Negative (-3.8)	Negative (-96.7)
7	52C1201	775	17	A203, A24; B18, B46; DR9, DR17; DQ2, DQ9	A2, A33; B46, B58; Bw4, Bw6 DR9, DR17; DQ2, DQ9	Negative	DP19 (1359)	Negative (13.8)	Negative (-249.9)
8	53S2335	7	82	A26, A33; B76, -; Bw6 DR15, DR17; DQ2, DQ5	A203, A34; B51, B62; DR4, DR15	**A203 (976)** Cw17 (2210)	**DRB1*0402 (558)** DP4 (2090)	Negative (7.2)	Negative (-80.1)
9	53P0950	10	0	A24, A29; B7, B35; DR10, DR12; DQ5, DQ7	A11, -; B75, -; Bw6 DR12, -; DQ5, DQ7	**A11 (4560)**	Negative	**Positive (98.4)**	**Positive (96)**
10	53R4143	51	3	A2, A11; B46, B75; DR12, DR15; DQ5,-	A2, A34; B46, B62; Bw6 DR12, DR15; DQ5,-	**B76 CREG B62 (5322)**	Negative	**Positive (55.8)**	**Positive (108.9)**

* Positive DSA and FCXM results are shown in bold.

risk group due to its high rate of false positive results.[17,29] However, the organ allocation in Thailand might affect our results. Patients in Thailand who are CDCXM positive are assigned as high-risk patients, and these individuals are prohibited from receiving kidney transplantation, while CDCXM negativity and FCXM positivity is not a contraindication for kidney transplantation.[18] This is due to the report that FCXM might be too sensitive, as also demonstrated in a previous publication.

In this study, there were 10 (3.7%) patients with an incidence of graft loss from AMR reported by the Thai Transplant Society. Interestingly, FCXM was significantly associated with clinical outcome at 1 year but not at 3 years after transplantation. This might be due to the natural history of AMR, which has a higher prevalence at the early-stage post-transplantation (1st year) compared to the later period. The correlation between DSA and FCXM in pre-transplanted patients has been demonstrated.[3,30,31] However, the correlation can vary significantly due to the difference in the cut-off value used.[32] In our study, with an analysis of the 10 patients with DSA available, we found that FCXM could detect positive results in these 4 samples with a value of MFI of the DSA test higher than 2,000. Six samples with DSA at MFI lower than 2000 could not be detected by FCXM, reciprocal to previous studies.[33,34] Interestingly, 3 patients (patient No. 3, 4, and 7 in Table 4) had negative FXCM in the pre-transplanted period but reported a low expression of HLA class II DSA experienced graft loss due to chronic AMR after 1 year post-transplantation. It is possible that FCXM negativity in pre-transplant serum in these patients might be due to the lower sensitivity of FCXM or the possibility that DSA develops later after transplantation.[35] Unfortunately, we did not have the serum of these patients after transplant to confirm this hypothesis.

Although positive FCXM particularly for HLA class I is significantly associated with graft loss from AMR within 1 year after transplantation in this study, there were a lot of false positive FCXM results, as high as 35.9%. Adding FCXM results to the process of patient selection might exclude a number of patients. Rather, we should perform FCXM along with CDCXM to monitor the graft outcome. There are guidelines for the detection and characterisation of clinically relevant antibodies in allotransplantation by different cross-matching methods from the British Society for Histocompatibility and Immunogenetics (BSHI)[36] that identify the risk for transplanted patients as follows. The patients with positive T and/or B cell FCXM, negative CDC, and positive DSA are classified as intermediate immunological risk patients. Only those patients with negative T and B cell FCXM, negative CDC, and negative DSA are classified as low immunological risk patients. Therefore, even with our unfavourable results, FCXM might be a useful method for monitoring the possibility of AMR after renal transplantation.

In conclusion, our results demonstrated that FCXM might have a limitation in Thailand renal transplantation, which might at least, in part, be due to the current organ allocation policy. Further studies of FCXM or studies of FCXM in other contexts will be needed for a solid conclusion to be drawn.

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Conflict of interest

None declared.

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