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Patients with immunological diseases or on peritoneal dialysis are prone to false positive flow cytometry crossmatch

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ABSTRACT

Despite implementation of virtual crossmatches, flow cytometry crossmatches (FCXM) are still used by many transplant centers to determine immunological risk before kidney transplantation. To determine if common profiles of patients prone to false positive FCXM exist, we examined the demographics and native diseases of kidney patients tested with autologous FCXM (n = 480). Improvements to FCXM and cell isolation methods significantly reduced the positive rate from 15.1% to 5.3%. Patients with native diseases considered 'immunological' (vasculitis, lupus, IgA nephropathy) had more positive autologous FCXM (OR = 3.36, p = 0.003) vs. patients with all other diseases. Patients who were tested using our updated method (n = 321) still showed that these immunological diseases were a significant predictor for positive autologous FCXM (OR = 4.79, p = 0.006). Interestingly, patients on peritoneal dialysis (PD) also had significantly more positive autologous FCXM than patients on hemodialysis or waiting for pre-emptive kidney transplants (OR = 3.27, p = 0.02). These findings were confirmed in patients who had false positive allogeneic FCXM. Twenty of 24 (83.3%) patients with false positive allogeneic FCXM tested with updated method either had immunological diseases originally or were on PD. Our findings are helpful when interpreting an unexpected positive FCXM, especially for transplantation from deceased donors.

1. Introduction

In the seminal paper published in 1969 [1], Patel and Terasaki reported positive complement-dependent cytotoxicity crossmatches (CDC-XM) predicted hyper acute rejection of kidney transplant. The presence of antibodies to donor HLA prior to transplantation is generally considered to be associated with high risk of antibody-mediated rejection (AMR) of kidney, pancreas, heart, lung transplants [2,3]. The CDC-XM detects cytotoxic antibodies but is subject to false positivity and false negativity. The sensitivity of the CDC-XM was improved by adding antihuman globin but still failed to predict early graft loss in kidney transplant while more sensitive flow cytometry crossmatches (FCXM) correlated better [4,5]. In the FCXM, patient serum was mixed with donor lymphocytes. Cell-bound IgG was detected with fluorescent-labeled anti-IgG secondary antibody. Later, pronase-treated lymphocytes were used to reduce false positivity caused by nonspecific binding with Fc receptors [6]. However, false positivity is still present [7,8], and may prevent patients from receiving otherwise low-risk life-saving organ transplantation. Common causes of false positive FCXM are autoantibodies, immune complexes, antibodies to the non-HLA antigen, or immune modulators [9,10] such as Rituximab, Alemtuzumab, and Anti-Human Thymoglobulin. There has not been a published study about the characteristics of patients who are prone to false positive FCXM. In this paper, we compared demographics and native diseases of patients with and without positive autologous FCXM. We then conducted exploratory analysis of our findings in a cohort of patients who had false positive allogeneic FCXM.

Abbreviations: AMR, antibody-mediated rejection; CDC, complement-dependent cytotoxicity; DSA, donor specific antibody; FCXM, flow cytometry crossmatch; HLA, human leukocytes antigen; HD, hemodialysis; PD, peritoneal dialysis; PE, pre-emptive transplantation; PRA, panel reactive antibody; XM, crossmatch

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2. Materials and methods

We reviewed consecutive autologous (February 2014 to April 2017), and false positive allogeneic FCXM (February 2014 to June 2018) performed in our laboratory for kidney or combined kidney transplants (i.e. kidney-liver, kidney-pancreas). All autologous FCXM in this paper were performed at the time of initial assessment for waiting list patients. Patients who were treated with Rituximab before serum collection were excluded in the study. Demographics and native diseases were collected for patients with positive or negative autologous FCXM, and patients with false positive allogeneic FCXM. An allogeneic FCXM was considered to be false positive when donor-specific antibodies (DSA) were unequivocally absent. Donors were typed for HLA-A, B, C, DRB1, DRB3/4/5, DQA1/B1 and DPA1/B1 in the low/intermediate level using reversed SSO (sequence-specific oligo probe) (One Lambda, Canoga Park, CA) or SSP (Sequence-specific primer) (Olerup, Sweden) kits. All 11 loci including DPA1/B1* and DQA* were included for unacceptable antigens and virtual crossmatches. SSP High-resolution typing was performed whenever necessary to rule out the presence of a possible allele-specific DSA. The presence of anti-HLA antibodies was determined with Luminex single antigen beads (SAB) (One Lambda, Canoga Park, CA). EDTA was used in all tested sera to remove the prozone effect [11,12]. Positive reactions were determined with consideration of median fluoresce intensity (generally MFI > 1000), cross-reactivity, epitope analysis, historical anti-HLA antibodies and sensitization history [13]. Panel Reactive Antibody (cPRA) was calculated using the Canadian cPRA calculator using an approach that was the same as assigning unacceptable antigens for all 11 loci [14]. A supplemental SAB panel was not used. Instead, an antibody to a non-tested allele was predicted by testing antigens sharing the same epitopes.

Three-color FCXM were performed with purified peripheral blood lymphocytes treated with pronase (Sigma Aldrich, St Louis, MO, USA, Catalog P5147, 0.6 mg/ml) according to a previously described protocol [6]. Initially, a conventional FCXM method [6] with Ficoll-purified cells was used (designated as “Old Method”). After May 2015, the cell isolation method was changed to a negative selection magnetic bead protocol using EasySepTM HLA WB Total Lymphocytes Enrichment kit on RoboSep^{TM} equipment (Stem Cell Technologies, Vancouver, BC, Canada). FCXM was also switched to a modified rapid Halifax protocol [15] but using tubes rather than plates (Designated as “New Method”) to preserve the benefit of efficient washing in tubes. Detailed protocols for the two methods are summarized in Table 3. The positive cut-off was defined using standard deviation (SD) calculations based on > 100 FCXMs with 20 antibody-negative sera and at least five donors cells. In this study, any positive (> 3SD above negative controls) or borderline positive (2.0–3.0SD above negative controls) FCXM found in at least one tested serum was recorded as positive. Because two different cytometers FC500 and Navios (both from Beckman Coulter) were used, cut-offs were defined using the MESF (Molecules of Equivalent Soluble Fluorochrome) bead units of patient sera subtracting the average of 3 negative sera (ΔMESF). All statistical analyses were performed with IBM SPSS version 25.

3. Results

480 patients waiting for a kidney, combined kidney/pancreas, or combined kidney/liver transplant were included in the cohort of autologous FCXM. Positive autologous FCXM were found in 41 (8.5%) patients, of which 9 (22.0%) were positive only for T cells, 20 (48.8%) only for B cells, 12 (29.3%) for both T cells and B cells (Table 1). In univariate binary logistic regression analysis, type of native diseases as a categorical variable was not significantly associated with positive autologous FCXM (p = 0.205). When a linear regression model was used, native diseases were not associated with relative positivity, measured with ΔMESF, for either T-FCXM (p = 0.774) or B-FCXM (p = 0.524). However, positive rates for autologous FCXM were much higher in patients with IgA nephropathy (18.2%) or autoimmunity diseases such as vasculitis/lupus (21.4%) than patients with other native diseases (6.3%–7.7%). Patients with autoimmunity diseases (n = 6) and those with IgA nephropathy (n = 5) were combined into a single group with immunological native diseases. In multivariate binary logistic regression analysis (Table 2), patients with immunological native diseases (20% positive rate) were more likely to have positive autologous FCXM [OR = 3.36 (1.51–7.51), p = 0.003] than patients with other native diseases (6.3% positive rate). Patients with PRA > 80% were also more likely to have positive autologous FCXM [OR = 2.04 (0.92–4.55)] than these with PRA < 80% but it is not statistically significant (p = 0.08). Gender, age, and whether patients were on peritoneal dialysis (PD) were not statistically significantly associated with positive autologous FCXM.

The positive rate of autologous FCXM was much higher for the old

Table 1
Autologous FCXM positive rate in kidney patients with different native diseases.

	Positive/Total (n)	Positive Rate (%)	T, B, T&B (n)
All	41/480	8.5	9, 20, 12
DM	12/160	7.5	3, 6, 3
HTN	4/54	7.4	1, 2, 1
AID	6/28	21.4	1, 3, 2
IgA	5/27	18.5	1, 2, 2
PCKD	4/60	6.6	1, 2, 1
GN	4/64	6.3	2, 2, 0
Hereditary	2/26	7.7	0, 1, 1
Others	4/61	6.6	0, 2, 2

Patients for kidney or combined kidney/pancreases, or combined kidney/liver transplant with native diseases of Diabetes Mellitus (DM), Hypertension (HTN), Autoimmunity diseases (AID) such as Vasculitis/lupus, IgA nephropathy (IgA), Polycystic kidney disease (PCKD), Glomerulonephritis other than IgA nephropathy (GN), Hereditary, and others. T: positive for T cell only; B: positive for B cells only; T&B: positive both T and B cells.

Table 2
Multivariate analysis for factors contributing to positive autologous flow crossmatches.

Variables	Positive, N (%)	Total	OR[1]	P-value
Total Auto FCXM	41 (8.5)	480		
Gender				
Female	17 (8.8)	194	1.29 (0.64–2.60)	0.47
Male	24 (8.4)	286	1	
Average Age	52.5	52.1	0.98 (0.96–1.01)	0.12
Test Method				
Old	24 (15.1)	159	3.28 (1.67–6.44)	**0.001**
New	17 (5.3)	321	1	
Native diseases				
Immunological[2]	11 (20.0)	55	3.36 (1.51–7.51)	**0.003**
Others[3]	30 (6.3)	425	1	
Dialysis				
PD[4]	14 (11.3)	124	1.63 (0.82–3.32)	0.18
Others[5]	27 (7.6)	356	1	
cPRA				
> 80%	11 (13.9)	79	2.04 (0.92–4.55)	0.08
< 80%	30 (7.5)	401	1	

P-value < 0.05 was highlighted in bold.
[1] Odd Ratio (95% confidence interval), multivariate binary logistic regression using SPSS.
[2] Immunological diseases include lupus, vasculitis, IgA.
[3] Other diseases include DM, HTN, PCKD, GN other than IgA, Hereditary, and others.
[4] Patients on peritoneal dialysis.
[5] Others include patients who are on hemodialysis or waiting for pre-emptive kidney transplantation.

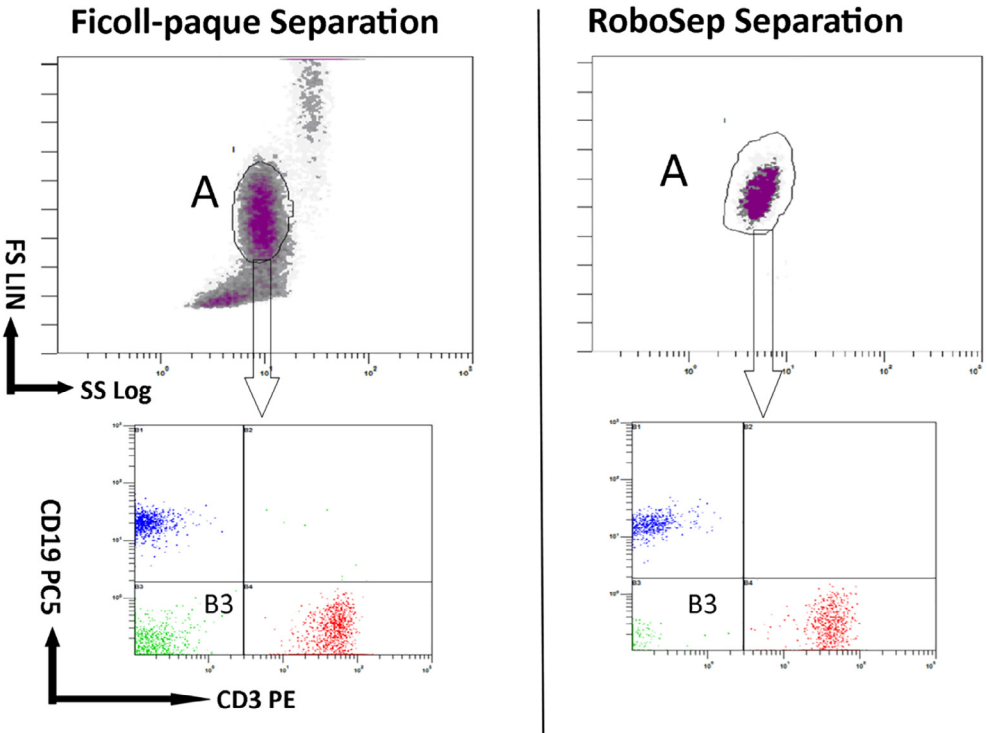
Table 3
Technical comparison of the old and the new methods for flow crossmatches.

	Before May 2015 (Old method)	After May 2015 (New method)
Cell Separation Methods		
	Blood, Ficoll-Paque	Blood, Robo-Sep
Pronase Treatment Procedures		
Concentration of pronase	0.6 mg/ml, 2 ml pronase for 6–15 × 10^6 cells	
Incubation	20 min @ 37 °C	15 min @ 37 °C
Washes	1 × 10 min	1 × 3 min
Prepare Cells for Flow cytometry		
Incubation methods	Tubes (Falcon 2063)	
Volume of Serum	25 µl	50 µl
Volume (number) of cells	25 µl (0.25 × 10^6)	
1st incubation	30 min @ Room temperature	20 min @ Room temperature
Washes	3× (3 min wash)	
Antibody Cocktail	30 µl FITC working solution + 5 µl CD3 + 5 µl CD19	
2nd incubation	30 min in the Dark @ RT	10 min in the Dark @RT
Washes	3 × (3 min wash)	1 × (3 min wash)
Suspension buffer	600 µl PBS	200 µl PBS
Flow cytometer	Beckman Coulter Navios	Beckman Coulter Navios or FC500

cell separation and FCXM methods (15.1%) than the new methods (5.3%) [OR = 3.28 (1.67–6.44), p = 0.001)]. In the new protocols (Table 3), incubation times for many steps were reduced, such as with pronase (20 min to 15 min), with serum (30 min to 20 min), with antibody cocktail (30 min to 10 min). Numbers of washes were also cut from three to one. Cells were suspended in 200 µl instead of 600 µl buffer when counted. Highly concentrated cells will be collected faster in the flow cytometer. At the same time, we also switched from Ficoll-Paque to faster and hands-free RoboSep methods for cell isolation. The viabilities of cells isolated with both methods were similar (96.9 ± 2.38% for Ficoll, 97.8 ± 2.85% for RoboSep; p = 0.28). However, when cells were purified using the new RoboSep method, contamination of granulocytes was dramatically reduced. As indicated in Fig. 1, the percentages of gated lymphocytes were much higher for cells isolated with RoboSep (78.7 ± 7.73%) than with Ficoll (39.21 ± 19.75) (p < 0.001). Proportions of cells which were neither T cells ($CD3^+$) nor B cells ($CD19^+$) were also significantly less in RoboSep isolated cells (6.53 ± 5.84%) than in Ficoll isolated cells (19.36 ± 10.05%) (p < 0.001).

Given the fact that FCXM and cell isolation methods significantly reduced the rate of the positive autologous FCXM, we examined if the same intrinsic features for positive autologous FCXM were still present with optimized methods (n = 321). In multivariate binary logistic regression analysis (Table 4), patients with immunological native diseases (15.8% positive rate) were still more likely to have positive autologous FCXM [OR = 4.79 (1.57–14.56), p = 0.006] than patients with other native diseases (3.9% positive rate). Interestingly, patients who were on peritoneal dialysis had significantly more (10.4% vs. 3.7%) positive autologous FCXM than patients on hemodialysis or waiting for preemptive kidney transplantation [OR = 3.27 (1.17–9.13), p = 0.024]. There were statistically insignificant trends toward more positive autologous FCXM in female patients [OR = 1.61 (0.54–4.83), p = 0.40] vs. male patients and cPRA > 80% vs. cPRA < 80% [OR = 1.65 (0.43–6.66), p = 0.45].

In light of these findings, we then reviewed positive allogeneic FCXMs that were predicted to be negative by the virtual crossmatch in deceased donor allocation or kidney paired donation. These patients were waiting for a kidney transplant or a combined kidney transplant with pancreas or liver. As described in the method, positive FCXM were considered as false positive after a comprehensive effort was made to



	Ficoll (mean±SD)	RoboSep (mean±SD)	p-value
Cell viability (%)	96.90 ± 2.38	97.80 ± 2.85	0.28
Lymphocytes gate A (%)	39.21 ± 17.70	78.70 ± 7.73	<0.001
Non-T/B cells gate B3 (%)	19.36 ± 10.05	6.53 ± 5.84	<0.001

Fig. 1. Comparison of cell quality for the two cell separation methods. Legends: representative flow dot plots for all cells (upper panel), and gated lymphocytes (middle panel) after cells were isolated with either Ficoll-paque (left) or RoboSep (right) methods. Lower table, mean and SD were calculated with 20 FCXM (10 patient cells for autologous FCXM and 10 deceased donor cells for allogeneic FCXM) for the two cell separation methods. P-value was calculated with Student's T-test.

Table 4
Multivariate analysis for factors contributing to positive autologous flow crossmatches performed with the new method.

Variables	Positive, N (%)	Negative, N (%)	Subtotal	OR[2]	P-value
Auto FCXM tested with new method[1]	17 (5.3)	304 (94.7)	321		
Gender					
Female	6 (4.8)	120 (95.2)	126	1.61 (0.54–4.83)	0.40
Male	11 (5.6)	184 (94.4)	195	1	
Average Age	49.3	52.2		0.98 (0.94–1.01)	0.18
Native diseases					
Immunological [3]	6 (15.8)	32 (84.2)	38	4.79 (1.57–14.56)	**0.006**
Others[4]	11 (3.9)	272 (96.1)	283	1	
Dialysis					
PD[5]	8 (10.4)	69 (89.6)	77	3.27 (1.17–9.13)	**0.024**
Others[6]	9 (3.7)	235 (96.3)	244	1	
cPRA					
> 80%	3 (5.9)	46 (94.1)	49	1.65 (0.43–6.66)	0.45
< 80%	14 (5.1)	258 (94.9)	272	1	

P-value < 0.05 was highlighted in bold.
[1] Only patients tested with new methods were included (n = 321).
[2] Odd Ratio (95% confidence interval), multivariate binary logistic regression using SPSS.
[3] Immunological diseases include lupus, vasculitis, IgA.
[4] Other diseases include DM, HTN, PCKD, GN other than IgA, Hereditary, and others.
[5] Patients on peritoneal dialysis.
[6] Others include patients who are on hemodialysis or waiting for pre-emptive kidney transplantation.

Table 5
Characteristics for patients with false positive allogeneic FCXM.

False positive allogeneic FCXM – N (%)	Old Method (n = 12)	New Method (n = 24)	All (n = 36)
Donor types			
Deceased donors	12 (100.0)	21 (87.5)	33 (91.7)
Live donors	0 (0.0)	3 (12.5)	3 (8.3)
Immunological native disease[1]	4 (33.3)	11 (45.8)	15 (41.7)
Peritoneal dialysis (PD)	4 (33.3)	14 (58.3)	18 (50.0)
Immunological native disease or PD	8 (66.7)	20 (83.3)	28 (77.8)
Female	8 (66.7)	12 (50.0)	20 (55.6)
cPRA > 80%	4 (33.3)	7 (29.2)	11 (30.1)
Re-transplantation[2]	2 (16.7)	3 (12.5)	5 (13.9)
Transplanted with false positive FCXM	5 (41.7)	11 (45.8)	16 (44.4)
False positive allogeneic FCXM on			
T cells only	8 (66.7)	4 (16.7)	12 (33.3)
B cells only	1 (8.3)	12 (50.0)	13 (36.1)
Both T & B cells	3 (25.0)	8 (33.3)	11 (30.1)
Autologous FCXM[3]			
Not Tested	4 (33.3)	0 (0.0)	4 (11.1)
Tested	8 (66.7)	24 (100.0)	32 (88.9)
Positive	7 (87.5)	13 (54.1)	20 (62.5)
Negative	1 (12.5)	11 (45.8)	12 (37.7)
Allogeneic FCXM with other donors			
Not tested	4 (33.3)	14 (58.3)	18 (50.0)
Tested	8 (66.7)	10 (41.7)	18 (50.0)
False positive- Yes	6 (75.0)	9 (90.0)	15 (83.3)
False positive- No	2 (25.0)	1 (10.0)	3 (16.7)

[1] Immunological native disease includes lupus, vasculitis, IgA.
[2] All 5 patients were waiting for second kidney transplantation.
[3] Sera tested in autologous FCXM were different from sera tested in allogeneic FCXM except for one patient.

exclude possible involvement of allele-specific DSAs or low titer DSAs underrepresented in the SAB test. Sixteen patients, including 5 patients with broad allo-sensitization (cPRA = 100% for 2 patients, 99% for 1 patient, 97% for 2 patients) were transplanted despite false positive allogeneic FCXM. No acute antibody-mediated rejection (AMR), detection of *de novo* DSA, or elevated incidence of delayed graft function were found in 1-month post-transplant. Absence of early adverse events indicates our approach of determining false positive allogeneic FCXM was robust. During the four years (2014–2018), 36 patients were found to have false positive allogeneic FCXM tested with either old (n = 12) or new methods (n = 24), with either deceased donors (n = 33) or live donors (n = 3) (Table 5). For patients whose sera were tested in allogeneic FCXM with deceased donors, the false positive rate is 9.9% (12/121) for the old method and 5.1% (21/412) for the new method. Fifteen (41.7%) patients had immunological diseases such as lupus, vasculitis, IgA nephropathy; 28 (77.8%) patients either had immunological diseases or were on peritoneal dialysis. When analyzing only those FCXM tested with the improved new methods, false positive allogeneic FCXM were found in 24 patients. Eleven of 24 (45.8%) patients had immunological diseases such as lupus, vasculitis, IgA nephropathy. Twenty of 24 (83.3%) patients either had immunological diseases or were on peritoneal dialysis.

4. Discussion

Although many centers have implemented virtual crossmatch, it still cannot predict a physical crossmatch such as the FCXM with both high sensitivity and high specificity at the same time [7,8,16]. A pre-transplantation XM, either CDC-XM or FCXM, is still essential in assessing immunological risk for kidney transplantation, especially in patients with positive anti-HLA antibodies. There have been many anecdotal reports that autoimmune diseases such as lupus were associated with false positive CDC-XM or FCXM. In this study, we found that other than patients with lupus, patients with vasculitis, IgA nephropathy, or those on peritoneal dialysis were also prone to a positive autologous FCXM. The findings were confirmed in patients who had false positive allogeneic FCXM with deceased or live donors. When tested with improved FCXM methods, 20/24 of these patients either had immunological diseases or were on peritoneal dialysis (PD). Three of the remaining four patients had a recent history of infections such as pneumonia, bronchopulmonary aspergillosis, or multiple infections such as tuberculosis and syphilis. Infection was not found in one remaining female diabetic patient with strong positive T cell but negative B cell allogeneic and autologous FCXM. She had a negative PRA, therefore the FCXM was easily determined to be a false positive.

Recently, IgA nephropathy has also been considered as an autoimmune disease caused by immune complexes of galactose-deficient O-

glycans on IgA1 and anti-glycan IgG autoantibodies [17,18]. It is possible that the O-glycans or other autoantigens are also on lymphocytes. IgG antibodies in patient's serum bind the autoantigens on the cell surface, then are recognized by the anti-human IgG antibody in the FCXM. The finding that PD patients are prone to false positive FCXM is intriguing. PD solutions inhibit NFκB/IL-12p70 pathway toward Th1 polarizing via acidic stress [19] or suppressing monocyte maturation [20], so Th2 immunity prevails in PD patients [21,22]. Th1 cells were found to be more prevalent than Th2 cells in patients who were on hemodialysis [23]. As a consequence of the humoral-promoting Th2 responses, overall IgG level or autoantibodies might be elevated in PD patients. Alternatively, a weakened Th1 response and/or the proximity to bacterial products of the gut in PD patients may increase the risk for infection such as peritonitis [24]. HIV infections were reported to be associated with false positive allogeneic FCXM [25–27]. However, our cohort did not include any HIV positive wait-listed patients, so possible contributions of HIV infection to false positive allogeneic FCXM cannot be evaluated in this study. In the autologous FCXM cohort, we did not collect data regarding infection history, which may be another important indicator for false positive FCXM.

Did patients with false positive allogeneic FCXM also have positive autologous FCXM? Autologous FCXM were performed for 32 patients with false positive allogeneic FCXM, usually at the time of initial assessment and using sera that were different from the ones used for the allogeneic FCXM. Positive autologous FCXM were found in 20 (62.5%) patients; while 12 patients had negative autologous FCXM but false positive allogeneic FCXM. Seven discrepancies can be explained with temporal differences in the sera used in allogeneic vs. autologous FCXM. Five false positive allogeneic FCXM were tested with the sera after patients started PD, while negative autologous FCXM were tested when patients were assessed for pre-emptive kidney transplantation (n = 4) or were on hemodialysis (n = 1). Two false positive allogeneic FCXM were tested after patients had infection episodes. The other two false positive allogeneic FCXM might be unconvincingly positive: one was borderline positive on T cells but negative B cells, another was only positive with one of two tested sera. In all 12 cases with false positive allogeneic FCXM but negative autologous FCXM, fluorescence bindings in "no serum" controls were weaker than those for cells incubated with negative control human serum without anti-HLA antibodies. So, we did not find false positive allogeneic FCXM solely caused by possible precoating of donor lymphocytes with donor autoantibodies. Eighteen of 36 patients who had false positive allogeneic FCXM, were also tested in allogeneic FCXM with other donors. Fifteen patients also had false positive allogeneic FCXM with multiple donors. In some instances, falsely positive allogeneic FCXM were found with 8 or 10 donors. In summary, our data indicate that most false positive allergenic FCXM in our study are caused by factors intrinsic to patients, rather than donors or donor/recipient combinations. Serum/cell combinations might contribute to some of weak false positive allogeneic FCXM, which were not consistently false positive with different donors.

The new FCXM method using the modified Halifax protocol and magnetic beads purified lymphocytes have significantly reduced false positive rates (from 15.1% to 5.3%). The incubation time is shorter in the new method, so there is less time for weak non-HLA antibodies to bind. Cell purification with RoboSep had substantially fewer granulocytes contamination than the old method using Ficoll and centrifuging. Interestingly, for positive autologous FCXM tested with improved method, contributions of high cPRA > 80% diminished, while contributions of PD & Immunological diseases increased. This suggests that optimized FCXM and cell isolation protocol reduces noise caused by technical imperfections; in consequence true intrinsic causes of false positivity become more potent. Pronase-treated cells were used in all FCXM in this study. Pronase is a crude mixture of nonspecific bacterial proteases, which can cleave the Fc receptors and many other surface proteins. Treating lymphocytes with pronase has been reported to dramatically reduce false positive FCXM, especially for B cells [6,15,28] and has been used by all HLA labs in Canada. However, many labs [25,26,29–31] reported that pronase treatment could cause false positive FCXM, especially on T cells. Pronase was reported to change the expression of HLA or other proteins on lymphocytes [32], so it may cause either false positive or false negative results. However, only 9/41 (22%) of positive autologous FCXM, and 12/36 (33%) false positive allogeneic FCXM in our study were positive on T cell but negative on B cells. So pronase treatment might explain some but certainly not all false reactivity found in our cohort. Recently, blockage of Fc receptors with the anti-CD32 monoclonal antibody 6C4 [26] or novel DSA-FCXM with HLA-capturing antibodies [33] was reported as alternatives to reduce false positive FCXM.

Is 5.3% false positive rate too high? If borderline positive FCXM were excluded from the analysis, the positive rate for autologous FCXM with improved methods is only 4.36% (14 of 321), which is much lower than 6.7%–10% false positive rate previously reported in another lab [8]. There were broad ranges of positivity, measured in ΔMESF, for positive autologous T-FCXM (mean = 2458; range = 814–9189) or B-FCXM (mean = 7142, range 1554–31762), and for false positive allogeneic T-FCXM (Mean = 2889, range = 919–10800) or B-FCXM (mean = 4209, range = 1402–11744). Shall the positive cutoff be raised to reduce the false positive rate? Based on proficiency tests from American Society of Histocompatibility and Immunogenetics (ASHI), our FCXM is relatively sensitive in Canada and USA. Many low titer, clinically relevant real DSAs can be detected with our FCXM, so we rarely see rebounding or "*de novo*" DSA in early post-transplantation. While it is difficult to achieve perfect sensitivity and perfect specificity at the same time, we prefer excellent sensitivity and fairly good specificity. Selecting patients with immunological disease/PD for autologous FCXM at the time of initial assessment will help decision making when an allogeneic FCXM is unexpectedly positive after a negative virtual crossmatch. However, caution shall be taken if there is a substantial gap between times of autologous FCXM at initial assessment and deceased donor offers, considering the long waiting times for some kidney transplant candidates. Specifically, a patient's dialysis status may change from pre-emptive/hemodialysis to PD, and many new infections might occur. Nonetheless, our finding of immunological diseases and PD is beneficial to interpret unexpected positive allogeneic FCXM in time-sensitive transplantation from deceased donors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.03.015.

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