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Website: www.ajts.org
DOI: 10.4103/0973-6247.214355

# Detection of T and B cells specific complement-fixing alloantibodies using flow cytometry: A diagnostic approach for a resource limited laboratory

Dharmendra Jain, Pranav Dorwal, Amit Pande, Neetu Tyagi, Simmi Mehra, Vimarsh Raina

## Abstract:

**BACKGROUND AND OBJECTIVES:** Various methods have been reported for the detection of antibodies in recipient sera, which can be human leukocyte antigens (HLAs) or non-HLA specific, complement- or noncomplement fixing, as well as donor T (HLA-Class-I) and/or B cell (HLA-Class-I and II) specific. These alloantibodies play a pivotal role in antibody-mediated renal transplantation rejection. Deposition of C4d in peritubular capillaries of a kidney biopsy is a marker of antibody-mediated rejection. The C4d flow-panel reactive antibodies (PRAs) are a screening method for HLA-specific and complement fixing antibodies. However, the method is limited by the lack of donor specificity.

**DESIGN AND SETTINGS:** Here, we present a new and simple flow cytometric method referred to as C4d-flow cytometry crossmatch (C4d-FCXM) for the detection of donor-specific (T and/or B cell) and C4d-fixing alloantibodies.

**RESULTS:** The method was applied in a series of clinical cases and judged to be useful. The method may limit unwanted deferral of the donor due to positivity in C4d Flow-PRA and/or FCXM and may be helpful in prediction of antibody mediated rejections. Furthermore, this method can provide information pretransplant in contrast to kidney biopsy and C4d evaluation done posttransplant.

**CONCLUSIONS:** We postulate that this method incorporates most of the features of all the available modalities (i.e., National Institute of Health-complement dependent lymphocytotoxicity, FCXM, cytotoxic FCXM and C4d-flowPRA) yet cost-effective and best suited for resource-limited laboratory/ies which is a common scenario in developing countries.

## Keywords:

Alloantibodies, C4d, flow crossmatch, human leukocyte antigen, renal transplant

Department of Pathology  
and Laboratory Medicine,  
Molecular Genetics  
Laboratory, Medanta-  
The Medicity, Gurgaon,  
Haryana, India

## Address for correspondence:

Dr. Dharmendra Jain,  
Molecular Genetics  
and Immunology  
Laboratory, Medanta - The  
Medicity, Sector-38,  
Gurgaon - 122 001,  
Haryana, India.  
E-mail: jain\_  
dharmendra2004@  
hotmail.com

Submission: 21-07-2016  
Accepted: 14-10-2016

## Introduction

Hyper-acute rejections in renal transplants may occur in presensitized recipients due to alloantibodies against human leukocyte antigens (HLA) acquired through previous transplants, pregnancy or blood transfusions.<sup>[1,2]</sup> Various methods for the detection of clinically relevant alloantibodies have been reported which include

National Institute of Health-complement dependent lymphocytotoxicity (NIH-CDC) test, donor-specific flow cytometry crossmatch (FCXM), cytotoxic FCXM (cFCXM), solid phase assays such as panel reactive antibodies (PRA), Luminex crossmatch (Lx-XM), C4d flow-PRA, single antigen bead assay (SAB) for donor specific antibody identification, and C1q-SAB assay. These methods are used by various

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**How to cite this article:** Jain D, Dorwal P, Pande A, Tyagi N, Mehra S, Raina V. Detection of T and B cells specific complement-fixing alloantibodies using flow cytometry: A diagnostic approach for a resource limited laboratory. Asian J Transfus Sci 2017;11:171-9.

transplant centers based on their experience and feasibility; and have their own merits and demerits in terms of sensitivity; and specificity to donor's HLA system [Table 1].

NIH-CDC is being widely used in developing countries and has the capacity of detecting the complement fixing antibodies; however, it has relatively low sensitivity and it is subjective.<sup>[3]</sup> On the other hand, FCXM is far more sensitive for donor B and T cells, but its specificity is limited by its inability to differentiate

HLA from non-HLA specific; and complement from noncomplement fixing antibodies.<sup>[4]</sup> This may lead to a patient being unduly denied/delayed a transplant. The PRA has potential of differentiation between HLA and non-HLA antibodies but lacks specificity to donor cells/antigens.<sup>[5]</sup> The Flow-PRA and Luminex SAB can be helpful in this regard as a virtual crossmatch, but again it does not differentiate complement activating from noncomplement activating antibodies.<sup>[6]</sup> Furthermore, its high cost makes its use limited in a developing country. Lx-XM has advantages of donor specificity,

**Table 1: Comparative analysis of different method available for the detection of alloantibodies**

Assay name	Sensitivity	Donor specificity	HLA and non-HLA differentiation	Complement and noncomplement fixing antibody differentiation	Interpretation	Cost	Remark
NIH-CDC	Low	Yes	No	Yes	Subjective	Low	Low titer antibodies cannot be detected
AHG-CDC	Intermediate	Yes	No	Yes	Subjective	Low	Increase in damaged uninterpretable lymphocytes due to additional incubation steps as stress factor
FCXM	High	Yes	No	No	Objective	Low	Advantage of T- and B-cell specificity. Dependent on cell quality
cFCXM	Intermediate	Yes	No	Yes	Objective	Low	Cytotoxicity due to complement versus injury to cells during processing, cannot be differentiated
Flow-PRA	High	No	Yes	No	Objective	High	Independent of cell quality but not cost effective
AMS-ELISA	Intermediate	Yes	Yes	No	Objective	Intermediate	Preparation of cell lysate affects the protein confirmation (antigens and antibody) which may affect the binding
Lx-XM	High	Yes	Yes	No	Objective	High	Preparation of cell lysate affects the protein confirmation (antigens and antibody) which may affect the binding
C4d-PRA	High	No	Yes	Yes	Objective	High	Independent of cell quality but not cost effective
C1q-bead assay	High	No	Yes	Yes	Objective	High	Independent of cell quality but not cost effective
SAB	High	Yes	Yes	No	Objective	Very high	Cost of donor HLA typing for virtual crossmatch
C4d-FCXM	High	Yes	No	Yes	Objective	Low	Cost effective. May help in early and pretransplant detection of AMR

NIH = National Institute of Health, CDC = Complement dependent lymphocytotoxicity, FCXM = Flow cytometry crossmatch, cFCXM = Cytotoxic flow cytometry crossmatch, PRA = Panel reactive antibodies, Lx-XM = Luminex crossmatch, SAB = Single antigen bead, HLA = Human leukocyte antigens, AMR = Antibody-mediated rejection, AMS = Antibody monitoring system, AHG = Anti-human globulin

but it is unable to differentiate between complement fixing and noncomplement fixing alloantibodies. Moreover, the protocol used in Lx-XM may lead to conformational change in the structure of HLA antigens during the preparation of cell lysate.<sup>[7]</sup> Although SAB assays are standard of care, however, in the developing world cost is a major constraint. Although C1q SAB assay has capability to detect the complement fixing alloantibodies, their use is limited due to the facts that SAB assay is prone to saturation, the denaturation of coated antigen may lead to nonspecific binding, interference by C1 can affect the result values and a high cost of the beads used.<sup>[8]</sup> Together, the above methods have strengths and weakness of their own and complementarity to each other, providing limited stand-alone information.

C4d is one of the complement split products and its deposition in the peritubular capillaries (PTC) in a kidney biopsy is now widely accepted as a marker for AMR in renal allografts.<sup>[9]</sup> This has been ratified by Banff schema on the classification of renal transplant rejection.<sup>[10]</sup> Sensitized patient may experience from 40% to 90% incidents of AMR.<sup>[11,12]</sup> On conventional treatment the AMR can cause significant graft dysfunction and may lead to an irreversible and permanent injury and eventually graft failure.<sup>[13]</sup> The fact that antibody-mediated or humoral rejection increases the risk of chronic rejection makes it important to predict the AMR at early stages.<sup>[14,15]</sup> Although the recent Banff classification describes the entity of C4d negative AMR, there are mixed reports of association of C4d with AMR in renal transplant, and therefore, the deposition of C4d in PTC continues to be an important marker for complement activation.<sup>[16]</sup> Therefore, C4d fixing antibodies detection has lot of advantages over the above-discussed tests. The C4d Flow-PRA test compensates the limitations of the previously discussed test methodologies by identifying the complement fixing HLA antibodies, but again it lacks donor specificity.<sup>[17]</sup>

None of these methods can detect C4d fixing alloantibodies which are specific to antigens presents on the donor's cell surface.<sup>[14]</sup> Therefore, we report a cost-effective assay, that is, C4d-FCXM, for detection of donor T (HLA-Class-I) and/or B cell (HLA-Class-I and II) specific, C4d fixing alloantibodies for the prediction of the AMR. It helps in the decision of donor deferral, which is important in countries like India, where most of the transplants are live and related.<sup>[18]</sup> This method also gives an added advantage of detecting C4d fixing donor-specific antibodies in a pretransplant scenario in contrast to the widely used kidney biopsy based C4d deposition evaluated only posttransplant.

## Materials and Methods

The separation of peripheral blood mononuclear cells was done as per the protocol described by Böyum, in 1968<sup>[19]</sup> with a minor variation as mentioned hereafter. The heparinized peripheral blood sample of donor was diluted with the equal volume of McCoy's 5A Media (HiMedia Laboratories Pvt Ltd, India) and overlaid it on 3 ml of Ficoll hypaque solution (Lymphoprep™, Fresenius Kabi Norge AS, for Axis Shield P.C AS, Oslo Norway) and centrifuged at 400 g for 30 min. On the completion of centrifugation, mononuclear cells layer was isolated and mixed with 15 ml of 37°C heated McCoy's 5A Media. The cells were then incubated for 30 min at 37°C, and washed with McCoy's 5A Media by three repeated steps of centrifugation at 600 g and re-suspending the cell pellet in 15 ml of McCoy's 5A Media. The cell pellet was finally mixed with 1 ml McCoy's 5A Media. The differential cell count was done, and the cell concentration was adjusted to 0.25 million cells per 50 µl sample in McCoy's 5A Media. The test (recipient/patient) serum was kept at 56°C for 30 min for heat inactivation and centrifuged at 20,000 g for 10 min for the removal of the immune complexes.

Then, 50 µl or 0.25 million donor cell suspension was added in all five tubes of negative, positive control, pooled positive control, and test sample tubes (in duplicate). Anti-HLA negative control (Amtgerichtsstra BAG Healthcare, Lich, Europe), Anti-HLA positive control (Life Technologies Corporation, USA), pooled positive control and test serum were added in respective tubes and incubated for 30 min. After incubation, 30 µl serum from a nonsensitized healthy male volunteer having normal C4 level (10–40 mg/dl) with normal CH50 activity was added in all tubes. The suspension was then incubated for 30 min. The cells were then washed with McCoy's 5A Media by three repeated steps of centrifugation at 1200 ×g and re-suspending the cell pellet in 4 ml McCoy's 5A Media. To the cell pellet, 20 µl of working dilution of FITC-conjugated C4dAb2 (Biomedica medizinprodukte & Co KG) and 10 µl of Anti CD22 PE (Beckton Dickinson, USA) and 10 µl of Anti CD3 PerCP (Beckton Dickinson, USA) were added. These were mixed and incubated for 30 min. The cells were washed with McCoy's 5A Media by three repeated steps of centrifugation at 1200 g and resuspended the cell pellet in 4 ml McCoy's 5A Media. The cell pellet was finally resuspended in 500 µl of cold sheath fluid (BD FACFlow™, BD Biosciences, USA). A pictorial representation of the C4d activation and C4d FCXM protocol is given in Figure 1. Acquisition and analysis was performed using FACSuite™ software in FACSVerse™ Flow cytometer (BD Biosciences, USA). A minimum of 2000 of gated B-cells were acquired in the already existing assay template saved in the flow

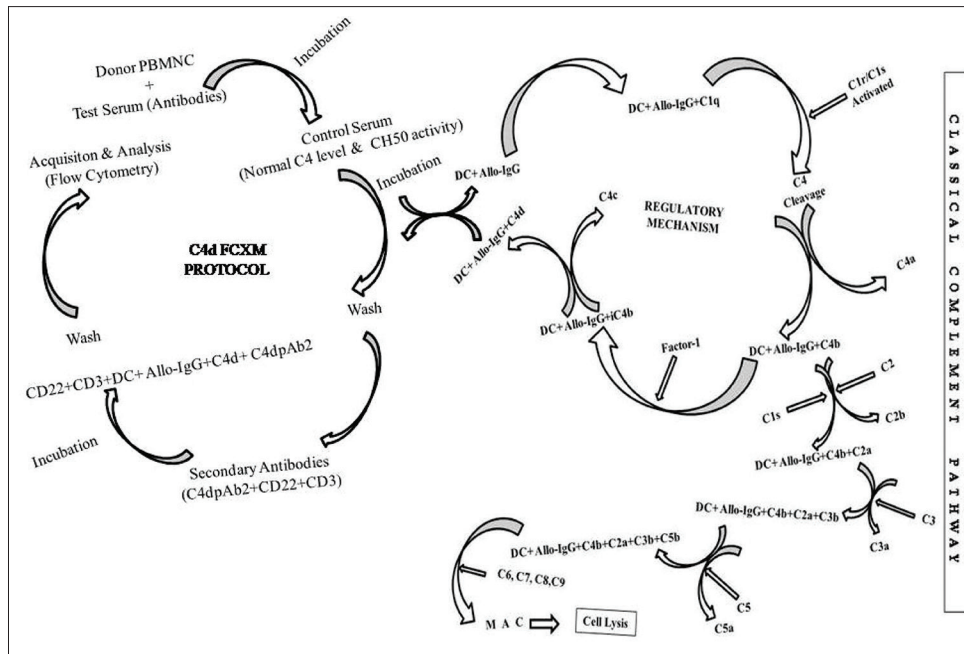


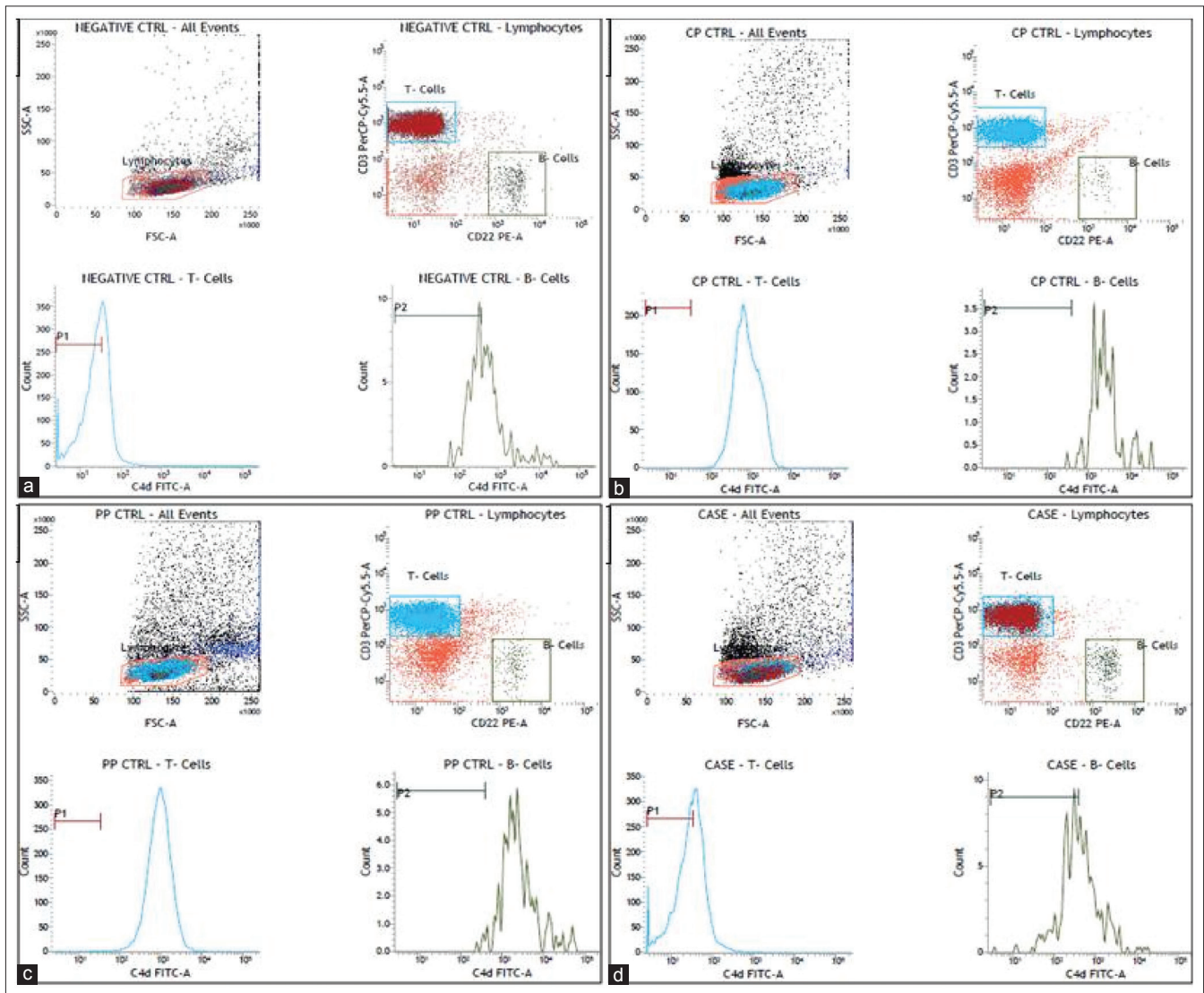
Figure 1: Pictorial representation of C4d activation and detection protocol in C4d-flow cytometry crossmatch protocol

cytometer. The cells of interest (i.e., lymphocytes) were gated on the forward-versus-side scatter dot plot. These cells were further sub gated on the second dot plot using antibodies for B cells (Anti-CD22 PE) and T cells (Anti-CD3 PerCP). A histogram was then created with cells gated on B cells and T cells each, and the median fluorescence intensity observed for FITC-conjugated C4dpAb2 for each population on a 1024 log scale. The controls were also analyzed in the same way and the values recorded. The cut off was calculated based on a set of normal nonsensitized control samples with the value calculated as three standard deviation of the mean. Cut off for our laboratory came out to be a shift of 27 for T cells and 180 for B cells vis-à-vis negative control. Reproducibility was confirmed by repeating the tests with the stored serum of the cases. With every run commercial positive control and pooled positive controls were used. Tests serums were run in duplicates. To circumvent the Fcy receptors interactions, an inherent properties of B-cell FCXM, the test was performed with both pronase treated and untreated donor cells. Although the median channel values of C4d FCXM were low with B-cells treated with pronase. A representative analysis graphs for C4d-FCXM are shown in Figure 2. NIH-CDC, FCXM, cFCXM, Lx-XM, and SAB assays were run as per the respective protocols provided by the manufacturer or the ones provided in the standard literature.<sup>[1,5-7,17,20]</sup>

## Results

The first case was that of a presensitized recipient of renal transplant. Due to the graft dysfunction, the kidney biopsy of renal allograft was tested for light

microscopy and immunofluorescence. The postoperative day (POD)-2 day biopsy showed patchy acute cortical necrosis. The C4d was diffusely positive in PTC in the preserved areas, whereas the POD-12 day showed negative C4d (Mouse Anti-Human C4d, Clone; 10-11, AbD Serotec, Canada) on immunofluorescence. The historic pretransplant serum was found to be negative for NIH-CDC, cFCXM, FCXM, and C4d-FCXM. Due to ambiguity for C4d on two consecutive biopsies, the posttransplant serum was tested and found to be positive for C4d-FCXM, which supports the diagnosis of AMR. The patient was then managed with therapeutic plasma exchange. The second case was of a nonsensitized young male, was on immune-suppression posttransplant. A 6-month posttransplant, the patient was diagnosed for herpetic esophagitis on esophageal biopsy and he was managed by decreasing immune-suppressions. Subsequently, an allograft renal biopsy was performed for light microscopy and immune-fluorescence due to the graft dysfunction, which showed acute cellular rejection (ACR) Stage-1A with calcineurin inhibitor toxicity; however, the C4d was negative in PTC. The patient was then managed with steroids. As the renal functions were not returning to be normal, to rule out the possibility of AMR, again an allograft renal biopsy was performed after 2 weeks for light microscopy and immune-fluorescence. The renal biopsy showed patchy positivity for C4d in PTC. The patient serum was also positive for NIH-CDC, FCXM, Flow-PRA, and the new method, that is, C4d-FCXM, for B-cells, which favors the diagnosis of AMR. The presence of donor specific antibody was confirmed in both of the above cases by SAB assay. There was another case of a young female



**Figure 2:** (a) Representing the analysis graph for a positive case for C4d-flow cytometry crossmatch. (b-d) The negative, positive (commercial positive control, Life technologies Corporation, USA), pooled positive controls, and case reactions, respectively

recipient whose NIH-CDC was negative, but donor was deferred due to positive FCXM for T cells during pretransplant workup. Subsequently, another unrelated donor (husband of recipient) was deferred as found to be negative in NIH-CDC but positive for donor B and T-cells specific alloantibodies in FCXM. She was positive for Flow-PRA and Lx-XM crossmatch but was negative for C4d FCXM. Thereby suggesting donor specific antibodies which are not complement fixing. The patient was planned for a renal transplant with unrelated donor by categorizing the patient in high-risk group. The retrospective analysis of two cases with Acute AMR were done those have no history of sensitization pretransplant. On posttransplant work-up, these were found to be positive for Flow-PRA, Lx-XM crossmatch, and C4d FCXM; however, weak positivity was seen in NIH-CDC and FCXM. The pretransplant historic serums were tested and were found negative for NIH-CDC and

FCXM. A prospective case of a young male with CKD-5 with no history of sensitization was tested for all the above-discussed technologies along with C4d-FCXM and found positive; therefore, the case was deferred for transplant. Finally, we assessed a nonsensitized recipient with a history of Ig-A nephropathy who was planned for a preemptive renal transplant at CKD-5. POD day 8 serum was negative for NIH-CDC and FCXM, but renal allograft biopsy showed vascular type, ACR. The patient was tried to be managed with steroid and therapeutic plasma exchanges; however, he was steroid resistant. The POD day-22 biopsy showed C4d negativity in PTC. The biopsy was reported with the impression of ACR. The POD day-22 serum of the patient was tested for the novel method, that is, C4d-FCXM and found to be negative which supports the histopathological findings. Table 2 summarizes the pre- and post-transplant test details of the cases.

**Table 2: Representation of clinically variant cases showing diagnostic utility of C4d-flow cytometry crossmatch**

Test	Case-1: 31/F	Case-2: 28/M	Case-3 30/F (donor-1)	Case-3 30/F (donor-2)	Case-4: 45/M	Case-5: 23/M	Case-6: 19/M	Case-7: 28/M
	Yes	No	Yes		No	No	No	No
<b>Pre-transplant</b>								
Nih-CDC	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative
cFCXM	Negative	Not done	T-cell positive	Negative	Not done	Not done	T & B - cell positive	Not done
FCXM	Negative	Not done	T cell positive	T & B - cell positive	Negative	Negative	T & B - cell positive	Not done
Flow-PRA	Not done	Not done	Class-i & ii positive		Not done	Not done	Class-i & ii positive	Not done
C4d FCXM	Negative	Not done	T-cell positive	Negative	Not done	Not done	T & B - cell positive	Not done
Luminex crossmatch	Not done	Not done	Class-i positive	Class-i & ii positive	Not done	Not done	Not done	Not done
Sab-class-i	Not done	Not done	Positive	Positive	Not done	Not done	Not done	Not done
Sab-class-ii	Not done	Not done	Positive	Positive	Not done	Not done	Not done	Not done
DSA	Not done	Not done	HLA-A*24, HLA-B*35	HLA-A*23, HLA-A*24:02, HLA-DRB1*09	Not done	Not done	Not done	Not done
<b>Post-transplant</b>								
NIH-CDC	Negative	B –cell positive	Transplant not done yet, but planned with donor-2 categorizing the patient as high risk		Negative	Negative	Transplant not done due to donor deferral	Negative
cFCXM	T & B - cell positive	B –cell positive			Negative	Negative		Negative
FCXM	T & B - cell positive	B –cell positive			Positive	Negative		Negative
Flow-PRA	Class-i & ii positive	Class-ii positive			Class-ii positive	Positive		Negative
C4d FCXM	T & B - cell positive	B –cell positive			B - cell positive	Class-i & ii positive		Negative
Luminex crossmatch	Not done	Not done			Class-ii positive	T & B - cell positive		Not done
Sab-class-i	Positive	Negative			Not done	Class-i & ii positive		Negative
Sab-class-ii	Positive	Positive			Not done	Not done		Negative
DSA	HLA-B*35, HLA-DRB1*10	HLA-DRB1*11:01, HLA-DRB1*11:04			Not done	Not done		Negative
						Not done		
Allograft biopsy								
Biopsy interpretation	Acute AMR	ACR//AMR-??	Not done	Not done	Inconclusive	Inconclusive	Not done	Acr
C4d (IF)	AMBIGUOUS	AMBIGUOUS	Not done	Not done	1	1	Not done	Negative

## Discussion

Unlike the west, majority of the renal transplants in India are live and related. In this study, we are reporting a new method of detecting complement activating donor T and/or B cell specific alloantibodies which have a great importance in the management of a renal transplant recipient, both pre- and post-transplant. This is, especially important in live related transplants where donor deferral is socio-economically an important issue. We discuss a variety of renal transplant cases representing a spectrum of renal transplant situations during pre- and post-transplants period, and role of C4d-FCXM method in the detection of complement fixing donor-specific alloantibodies.

There is an enormous improvement in the outcome of the renal transplant due to the availability of advanced

immune-suppressive therapies which help in the prevention of cell-mediated rejection and subsequently to the longer life of the renal allograft.<sup>[14]</sup> Unfortunately, there is always a risk of antibody mediated rejections which can only be subsidized by supportive therapies such as steroids and therapeutic plasma exchanges; however, steroid resistant AMR still remains an unsolved problem. The diagnosis of AMR has been discussed previously and there is a concrete guidelines available (Banff classification). Morphologic evidence of acute tissue injury, immuno-pathologic staining for C4d in PTC, and presence of circulating antibodies to donor HLA or other antigens expressed on donor endothelial cells are main criteria to confirm the diagnosis of AMR.<sup>[10]</sup> Initially, detection of alloantibodies in a recipient serum was considered a key factor for deferral of a live-related transplant, irrespective of specificity of these alloantibodies to donor HLA antigens or those being

complement activating. Later on, it was found that only HLA specific, complement activating alloantibodies are critical in a renal transplant and hence, screening a recipient for HLA specific antibodies was understood to be sufficient for avoiding AMR in renal transplant.<sup>[21]</sup> Keeping the fact in mind that the cytotoxic effect of an activated complement pathway is ultimately responsible for an AMR, the complement activating alloantibodies are now accepted to be one of the factors causing AMR. C4d emerged as a useful biomarker for the diagnosis of an AMR in renal transplants; however, there are conflicting reports about its usefulness. C4d is produced in the process of complement activation and regulation of C4. The C4d can be produced by classical as well as the mannose binding lectin complement activating pathways via a regulatory mechanism.<sup>[22,23]</sup>

As mentioned, a variety of methods is available which can detect these alloantibodies and help in the management of renal transplant. These methods have their own advantages, disadvantages and complementarity to each other when used for the diagnosis of AMR and subsequently to the management of a renal transplant. NIH-CDC is a standard method, however; there is always an associated risk of primary and memory alloantibody response in nonsensitized and presensitized recipient of transplant, respectively.<sup>[3]</sup> FCXM assay is extremely sensitive however there may be cases where the results for B-cells are affected by high background due to nonspecific binding of IgG to Fc receptors. It can be overcome by the use of proteolytic enzyme (pronase) making the assay more reliable, sensitive, and specific;<sup>[24]</sup> however, in this study, the overall results were unaffected with both pronase treated and untreated cells. The added advantages of using beads in IgG flow-PRA is to provide HLA specificity and differentiation between Class-I and Class-II antibodies, but it lacks donor specificity.<sup>[25]</sup> C4d flow-PRA has an advantage over other technology for detecting the HLA-specific, complement fixing alloantibodies, but it lacks the specificity to the donor's antigen.<sup>[15]</sup> The C1q SAB assay has been proven to be useful for the identification of the complement-fixing alloantibodies specific to HLA antigens coated on the beads. However, uses of beads coated with HLA antigens are costly and a limiting factor for a resource limited diagnostic settings, especially in developing countries. In such cases, the donor specificity of complement activating antibodies can easily be done using the donor's cells instead of the beads. Moreover, the C1q SAB assay requires an additional instrument like luminex which might not be available, while the proposed new assay can be done with a flow cytometer which is widely available in the majority of diagnostic hematology laboratories. Due to its potential of detecting the complement-fixing, donor

T and/or B cell specific alloantibodies, this new method, that is, C4d FCXM helps in decision of donor deferral or diagnosis of AMR.

The use of cell viability dyes such as 7-AAD in cFCXM may help in simultaneous detection of antibody binding and cytotoxicity produced in an FCXM, however, the cytotoxicity due to complement versus injury to cells while processing, cannot be differentiated.<sup>[26]</sup> We have observed some degree of B-cell death in the negative control. Furthermore, cFCXM is an indirect assessment in terms of its dependence on the cell death caused by the complement activation while the C4d-FCXM directly detects the activated complement.

In the current prototype cases cited, first two cases were of AMR, where initially there was a dilemma of AMR versus ACR as the result of histopathological finding of allograft biopsy was not sufficient for confirmation. Although, the earlier technologies such as FCXM were positive but were insufficient to confirm it as complement-mediated rejections. Although the second case was only positive for B-cells on NIH-CDC, cFCXM, FCXM, and C4d FCXM, it was valuable in diagnosing AMR. Various contrasting studies have reported the association of B-cell crossmatch<sup>[27]</sup> with graft survival.<sup>[28]</sup> The T-cell positivity is, however, found to be extremely relevant even with a negative CDC crossmatch<sup>[29]</sup> Flow-PRA showed the presence of HLA-specific antibodies positive in both the cases, but then its specificity to donor and complement pathway was in question. Furthermore, the alloantibodies specific to HLA detected by Flow-PRA do not always correlate with C4d positivity in the allograft biopsy.<sup>[30]</sup> Therefore, it is important to discriminate between the presence of complement fixing versus noncomplement fixing antibodies as done in the present case. The positivity on C4d-FCXM method confirmed the diagnosis of AMR due to donor B cell specific, complement fixing, alloantibodies for these cases. The current findings are similar to what seen by Bartel *et al.* wherein the C4d fixing alloantibodies were found to be associated with the capillary C4d deposition in renal allograft.<sup>[31]</sup>

The third case was of a deferred pretransplant donor; wherein, the recipient serum was confirmed to be positive for donor T and B cell specific; complement fixing, HLA alloantibodies. This was helpful in pretransplant prediction of a possibility of an AMR. The negative NIH-CDC in all the above three cases indicates low titer HLA antibodies. It has been reported that there is good correlation between the presence of C4d staining and circulating alloantibodies, but the presence of C4d without the histopathological investigation on allograft biopsy is insufficient to make a diagnosis of AMR.<sup>[10]</sup> However, such early and pretransplant prediction of

AMR may help the clinician to counsel the patient more effectively and for proactive management, if the transplant is proceeded categorizing such patients in a high-risk group.

The pretransplant negative and posttransplant positivity for alloantibodies in next two consecutive cases of acute AMR discussed indicate development of *de novo* alloantibodies posttransplant.<sup>[32]</sup> The sixth case of deferral for transplant supports the correlation of the available technologies with C4d-FCXM for detecting complement fixing donor specific alloantibodies. The last case was a clear indication of correlation of the C4d-FCXM and the histopathological findings.

The drawback of the current study was that the results were not compared with the previously described assays such as C1q bead assay and C3d bead assay which can be done in future studies. Though the new method detects the presence of alloantibodies with greater sensitivity and specificity, it fails to assess specifically the damaging effect of antibodies on local tissue that can only be confirmed through tissue biopsies. Studies considering the situations like transplant accommodation are required; wherein, the presence of alloantibodies have not always been correlated with histopathological indications of rejection of allograft.<sup>[33]</sup> There is a need to extensively study the outcome of renal transplant performed with permutation and combination of all three parameters, that is, alloantibodies specific to HLA, complement activation pathways and the donor's antigens exclusively.

## Conclusion

The presented new approach of detection of donor T and B cell specific, complement activating alloantibody is a sensitive, cost-effective approach based on flow cytometry that can be used in resource-limited diagnostic settings for pre- and post-transplant prediction of AMR.

## Acknowledgment

We would like to thank the team members of the Division of Nephrology, Medanta Kidney and Urology Institute, Medanta, The Medicity for their support.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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