Society for Heart Failure and Transplantation

SOCIETY FOR HEART FAILURE AND TRANSPLANTATION'S

THE REVIVAL

Promoting Academics to Improve Clinical Outcomes.

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EDITOR'S NOTE

Dr Manoj Durairaj

Heart Transplant Surgeon, MS, MCh. (AIIMS, New Delhi), FACC.

President of the Indian Association of Cardiovascular-Thoracic Surgeons.

Director, Marian Cardiac Centre and Research Foundation.

Program Director, Department of Heart and Lung Transplantation, Sahyadri Hospitals, Pune.

Dear Readers of The Revival,

Greetings on behalf of the Editorial board. This issue of The Revival carries the 3rd part of the 4 article series on Heart Transplant Immunology by Dr Chintan Sheth.

Dr. Chintan Sheth's insightful article takes us into the intricate realm of transplant immunology, particularly focusing on the clinical implications. With a meticulous exploration of pre-transplant immunology workup procedures, including HLA antibody screening methodologies and their interpretation, Dr. Sheth provides clinicians with invaluable guidance for assessing immunological risks and optimizing transplant outcomes. By elucidating the significance of Calculated Panel Reactive Antibody (cPRA) testing and its implications for donor selection, the article empowers practitioners to make informed decisions regarding transplant compatibility. Furthermore, Dr. Sheth navigates through the complexities of immunological workup at the time of transplant, offering a comprehensive overview of cross-matching techniques and their diagnostic implications. Overall, this editorial offers a comprehensive synthesis of Dr. Sheth's article, emphasizing its importance in advancing clinical practice and enhancing patient care in the field of transplant medicine.

Wishing our dear readers a Happy Reading!

Dr Manoj Durairaj
Editor "The Revival"

SUB EDITOR

Dr Talha Meeran

MBBS, MD, FACC,
Consultant Cardiologist,
Dept of Advanced Cardiac Sciences and Cardiac Transplant,
Sir HN Reliance Foundation Hospital, Mumbai

Dear Colleagues,

This edition of REVIVAL is third in a series of four review articles by Dr Chintan Seth focusing on transplant immunology. The current issue of REVIVAL talks about the basics of immunological testing prior and immediately following the transplant. Of particular note are the sections pertaining to the relevance of cPRA in our Indian setting, false positive and false negative testings results for PRA and the importance of single antigen bead assays and virtual cross match. The table at the end summarizing the various possible clinical scenarios of immunological testing outcomes with their actual clinical interpretation is an important highlight.

Sincerely,
Dr Talha Meeran
Sub Editor "The Revival"

Society Reg.No.: EKM/TC/186/2013,

Society secretariat: Address 36/117, 2nd Floor, Lisie Hospital Road, Kottecanal Junction, Kochi 682018. **Ph.:** 025750048, **Email:** infoshft@gmail.com, www.sfhft.org

Please call or write to us: Call: 9822322072, 9167048815, manojdurairaj@hotmail.com, talha.meeran@gmail.com

Link for membership, http://www.sfhft.org/application.html

Special thanks to Dr Chintan Sheth for authoring this month's article.

Designed by Maithili Kulkarni

Dr Julius Punnen

Sr. Consultant Cardiothoracic Surgeon, Narayana Health, Bangalore

Dear Members and Colleagues,

The success of heart transplant largely depends on a meticulous pre-transplant immunology workup, particularly the assessment of human leukocyte antigen (HLA) antibodies in the recipient. The third part of the transplant immunology review by Dr. Chintan Sheth, an expert Cardiac and Heart & Lung Transplant Anesthesiologist and Cardiac Critical Specialist, published in this issue of the Revival, delves into the significance of HLA antibody screening and the diverse techniques employed in this critical pre-transplant evaluation and the immunological evaluation necessary at the time of transplantation.

HLA antibody levels play a pivotal role in determining the success of organ transplantation, as preformed antibodies reduce the likelihood of finding a suitable donor, and can also lead to allograft failure. Almost one-third of waitlisted patients exhibit anti-HLA antibodies, primarily due to events like pregnancy, blood transfusions, and prior transplantation. To assess sensitization, various screening methods are employed, notably complement-dependent cytotoxic (CDC) antibody screening and solid-phase antibody screening.

The CDC test involves mixing recipient serum with donor lymphocytes, rabbit complement and dye, providing a semi-quantitative measure of cytotoxicity based on the degree of lysis of donor lymphocytes. Solid-phase antibody screening methods, on the other hand, employs enzyme-linked immunosorbent assay (ELISA) platforms or microbead platforms, instead of donor lymphocytes, to detect HLA antibodies. Conventional flow cytometry uses beads coated with different multiple HLA antigens on each bead while, in the single antigen bead (SAB) assay with "Luminex", each bead is coated with only one type of HLA antigen. Both techniques use fluorescent conjugated anti-IgG antibodies to detect anti-HLA antibodies and the amount of antibody is commonly reported as Median Fluorescence Intensity (MFI). The SAB assay has gained popularity due to its precision in identifying distinct antibody specificities. Generally, an MFI value >2000 is considered to be a significant level of antibodies; while a value > 10,000 that is donor specific, is very likely to result in allograft rejection. Different laboratories employ different MFI cut offs to give a positive result. The panel of reactive antibodies (PRA) test, includes a panel of HLA molecules. However, commercially available kits do not include all HLA class I and II molecules, and importantly, they do not represent the HLA molecules prevalent in the Indian population. Calculated PRA (cPRA) uses a computerized database to report the likelihood of finding a suitable donor, based on the degree of and specificity of anti-HLA antibodies in a given transplant candidate. However, cPRA calculators are not yet available for the Indian population.

At the time of transplant, various cross-matching techniques, including cell-based CDC cross-match, solid-phase flow cytometry cross-match (FCXM), and virtual cross-match, are employed to ensure compatibility and avoid hyperacute rejection. CDC has the advantage of only detecting complement binding antibodies, while the FCXM is very sensitive and may detect antibodies that may not be complement binding, and hence may not be clinically relevant.

Virtual cross-match involves utilizing the SAB assay and donor's HLA type to ensure no donor-specific antibodies are present. However, donor HLA type is not always known ahead of transplant, which may limit the ability to employ this technique.

In conclusion, this article highlights various immunological testing methodologies, their advantages and limitations, and sheds light on the need for a comprehensive evaluation considering prior sensitization history, antibody strength, and specificity. Advancements in immunology workup, particularly HLA antibody screening, have significantly contributed to enhancing the success rates of organ transplantation. As technology continues to evolve, ongoing efforts to address challenges in result interpretation, false positives/negatives, and population-specific data will further refine pre-transplant assessments, ensuring better outcomes for transplant recipients.

With warm regards,

Dr Julius Punnen

President, Society for Heart Failure and Transplantation

TRANSPLANT IMMUNOLOGY-
CLINICAL IMPLICATIONS (PART - III)

ABOUT THE AUTHOR

Dr. Chintan Sheth

DA, DNB, FICA, FTEE

Consultant Cardiac and Heart Transplant Anesthesiologist, Marengo CIMS Hospital, Ahmedabad, Gujarat

Working as a Consultant Cardiac and Heart & Lung Transplant Anesthesiologist And Cardiac Critical Specialist at Marengo CIMS Hospital for 12 years.

Successfully managed 45 Heart transplant recipients over 6 years.

Completed structured observer ship for heart and Lung Transplant and MCS for 1 month at UPMC under Dr. Robert Kormos, Pittsburgh and Allegheny general Hospital, Pittsburgh in 2016.

Had Topped the FTEE (Fellowship in Transesophageal Echocardiography) exams in 2016 with Prof. Dr. Kumar Belani award.

Did DA (Diploma Anesthesia) from Stanley Medical College, Chennai in 2008 and DNB anesthesia from Narayana Hrudayala, Bangalore in 2010.

Fellowship in Cardiac Anesthesia (FICA) from Narayana Hrudayalaya, Banglore under Dr. Muralidhar Kanchi in 2011.

Continued from Part II

Immunology work up

Pre-transplant

HLA Antibody Screening

At pre-transplant evaluation, the recipient's HLA antibody levels are estimated to determine the level of sensitization. Almost a third of patients who are waitlisted for transplantation may have a degree of anti-HLA antibodies detected. The usual route for sensitisation towards HLA antigens occurs in three instances; pregnancy, post blood transfusion and prior transplantation. Preformed antibodies increase the chances of immunological failure of the allograft by causing positive crossmatches and, thereby, result in the exclusion of donors.

There are different methods used for HLA antibody screening as shown below.

1. Complement Dependent Cytotoxic (cell-based) antibody screening

In CDC test, recipient serum is mixed with isolated viable donor lymphocytes along with rabbit complement and eosin vital dye. When recipient serum has antibodies that bind to the cell surface of donor lymphocytes with adequate density then rabbit complement activate the complement system for the lysis antigen-antibodies bound donor lymphocytes9. Further, vital dye will uptake by the lysed cells. (Figure: 7). The degree of cytotoxicity is expressed as percentage of lysed donor lymphocytes (% of dead cells) or score system (0 to 8). (table 1)

% of dead cells	Score	Interpretation
--	0	Not readable
0-10	1	Negative
11-20	2	Doubtful negative
21-50	4	Weak positive
51-80	6	Positive
81-100	8	Strong positive

Table1: Scoring and interpretation of CDC report

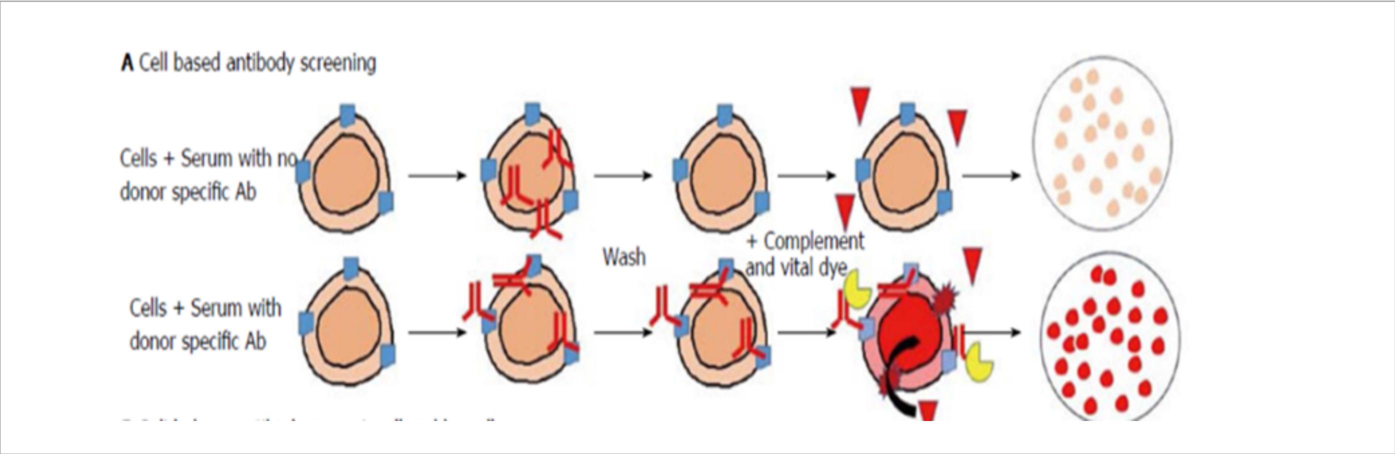

Figure 7: Complement Dependent Cytotoxic test

Figure 7 Courtesy: Althaf, Mohammed Mahdi & El Kossi, Mohsen & Jin, Jon & Sharma, Ajay & Halawa, Ahmed. (2017). Human leukocyte antigen typing and crossmatch: A comprehensive review. World Journal of Transplantation. 7. 339-348. 10.5500/wjt.v7.i6.339.

2. Solid phase antibody screening

2.1 Enzyme-linked immunosorbent assay (ELISA) platform

In this method, purified HLA molecules are applied to enzyme-linked immunosorbent assay (ELISA) platforms that will bind individually to HLA antibody after the addition of recipient serum. Enzyme conjugated antibodies to IgG (human) is then added to detect the presence of HLA antibody in the serum which is bound to the antigen10,11. Detection is performed by optical density reading. This method is now not in commonly used.

2.2 Micro-bead platform/single-antigen beads

This method employs soluble or recombinant HLA molecules on beads instead of lymphocytes targets - as lymphocytes present both HLA as well as non-HLA molecules.

There are 2 techniques uses beads as platform.

1. Conventional flow cytometry
2. Single antigen bead assay on Luminex platform

Both techniques use fluorescent conjugated anti IgG to detect anti HLA antibodies against donor and commonly reported as Median Fluorescence Intensity (MFI). Beads are coated with pooled donor Class I and II HLA antigens. The variants of these methods are:

2.2.1 Conventional flow cytometry

Uses beads coated with different multiple HLA antigens on each bead while is SAB with Luminex each bead is coated with only 1 type of known HLA antigen. Patient serum is mixed with these beads and antibodies present in patient's serum binds with these HLA antigens on beads. Fluorescent conjugated anti IgG is added to this which bind with patient's HLA antibody and which is read by flow cytometry machine with Median Fluorescence Intensity (MFI). These estimate PRA by the proportion of positive beads. (Figure: 9). Higher the binding of antibodies gives higher MFI value and generally MFI >2000 is considered significant level of presence of antibodies. Different laboratories may have different MFI cut off to give positive result.

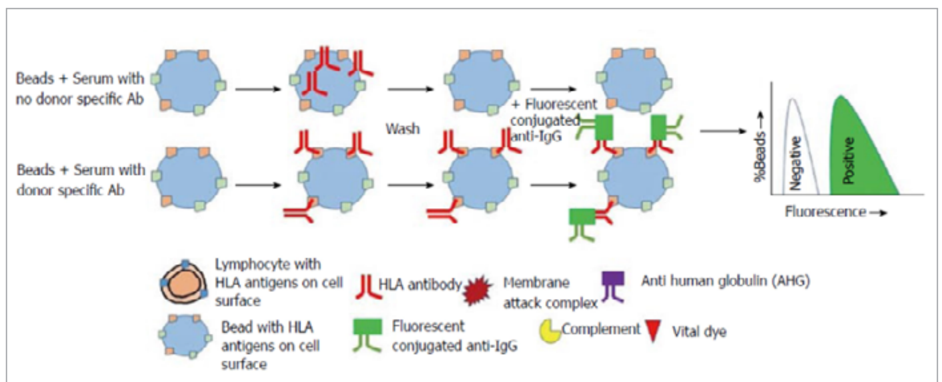

Figure 8: Conventional Flow Cytometry test

Figure 8 Courtesy: Althaf, Mohammed Mahdi & El Kossi, Mohsen & Jin, Jon & Sharma, Ajay & Halawa, Ahmed. (2017). Human leukocyte antigen typing and crossmatch: A comprehensive review. World Journal of Transplantation. 7. 339-348. 10.5500/wjt. v7.i6.339

2.2.2 Luminex Fluorocytometry

The second, which is more popular method is the use of the Luminex® fluorocytometry which as described earlier, uses single bead coated with single specific HLA antigen. Luminex utilizes two lasers, one of which excites the fluorochrome in the bead and the other laser exciting the detection antibody. The first readout therefore identifies the unique signal of the bead and hence the specificity of the bound HLA molecule, while the second readout indicates if antibody is bound to the specific HLA molecule (Figure: 9). SAB are individually coated with a single HLA antigen and yield a list of distinct antibody specificities.

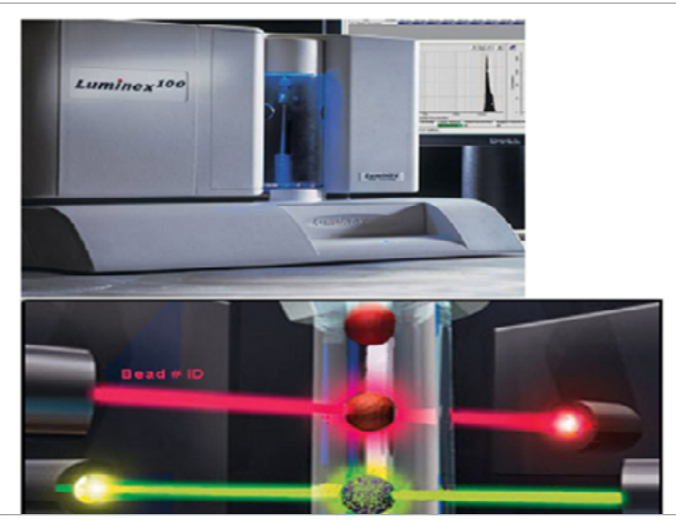

Figure 9: the top panel shows the luminex instrument. The bottom panel shows two lasers in the luminex instrument. One red laser identifies bead with attached HLA molecule and second green identifies the HLA antibody.

Figure 9 Courtesy: Tait BD (2016) Detection of HLA Antibodies in Organ Transplant Recipients – Triumphs and Challenges of the Solid Phase Bead Assay. Front. Immunol. 7:570. doi: 10.3389/fimmu.2016.00570

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1. PRA test has been extremely useful in providing information about the sensitization of a recipient. However, **the panel does not represent all HLA class I and class II molecules.**
2. The **HLA antibody specificity is not known** as there are several antigens in each well. So, PRA can just give us the idea about percentage presence or absence of class I or class II HLA antibodies but not exactly the which antibodies are present e.g. HLA DRB1*01:01. This exact specific antibody can be found out by Single antigen bead test assay.
3. Each population will have a different demographic prevalence of particular antigens, and so the PRA test panel constituents will differ from country to country. **But unfortunately we don't have PRA kit specific for Indian population. So all the Indian laboratories use foreign PRA kit and that's why it is utmost important to gather data as per Indian donor population to generate our own PRA kit and also for Calculated PRA (cPRA). At present we don't have Indian cPRA.**
4. Significant **false positive** results can be produced due to non-HLA antibodies, autoantibodies and nonspecific IgM antibodies.
5. **False negative** results are possible as this is purely complement dependent that requires higher antibody titres to be activated. The lack of a complement activation simply due to low titres allows a true antibody to be hidden.
6. It also detects both IgG and IgM HLA antibodies in addition to autoantibodies and non-HLA antibodies against other cell surface determinants which have no relevance in organ transplantation.
7. If PRA is 0% - No risk of hyperacute rejection but still recipient can have acute rejection as PRA kit does not detect all the HLA antibodies and also non HLA antigens can also elicit rejection.
8. If PRA is > 0%, at time of transplant, ideally HLA cross match must be done as donor may have same antigen against which recipient has pre-formed antibodies (which is detected by PRA) and that can cause rejection. In general, not every centre has access to get the HLA cross match report in time before transplant in that case PRA < 10% can undergo transplant with induction immunosuppressants with still some risk of rejection.

Calculated PRA (cPRA)

Still not available in India. Flow cytometry or Luminex assay is run against a fixed panel of HLA antigens from past organ donors in a large computerized database and the percentage of specific unsuitable antigens reported. This is called as calculated PRA (cPRA) in the US and calculated Reaction Frequency (cRF) in UK. PRA/cPRA measure the level of HLA sensitization, the recipients' likelihood of a positive cross-match but cannot determine the presence of antibodies against a specific donor. cPRA > 50% - need for desensitization

CLINICAL PEARL

Single antigen beat test has good sensitivity and specificity but cannot detect all the possible known HLA antibodies as number of coated beads are limited. Therefor it is not commonly used for HLA screening. On the other hand, CDC lacks specificity but can detect large group / panel of HLA antibodies and therefor it is generally used for screening.

Immunology work up at the time of transplant

1. Cell based CDC cross-match

The technique is similar to the one described above (PRA), except that for cross-match, actual donor's cells, instead of pooled donor cells are used. The cross match is performed at 4°C, 22°C and 37°C to detect warm and cold reactive antibodies.

It is reported qualitatively (positive or negative) or semi-quantitatively either as percentage of dead cells to live cells (0-no dead cells, 2-20% lyses, 4, 6, 8-80% lyses) or repeated dilutions (titres)

2. Solid Phase Flow Cytometry Cross-Match (FCXM)

In which recipient's serum is mixed with donor lymphocytes and incubated with fluorescein-labelled monoclonal antibodies against human CD3, CD19 and IgG. These anti human antibodies bind with donor T and B cells to evaluate the donor specific IgG antibody (DSA). FCXM is more sensitive than CDC and detects complement dependent and complement-independent IgG DSA. Although a median channel shift (MCS) is varying from the diagnostic centres. Mostly the MCS for T cells lies between 25 to 100 and for B cell 100 to 200. In about 15% cases, the CDC is negative with a positive FXCM, which most commonly indicates low antibody titres. The strength of antibody detected and prior antigen exposure history should also be considered, using such grafts may cause rejection and early graft loss and therefore should be closely monitored.

3. Virtual cross match

SAB is useful for virtual cross match when real time CDC is not available. So in that case donor's HLA typing gives idea about HLA antigens present in donor and SAB test gives idea about HLA antibodies in recipient's blood and these reports can be matched for presence or absence of specific antibodies against donor antigen. In that case though recipient's PRA is quite higher (for example 50%) but if no any antibodies against the antigens present in the specific donor sample, transplant can be safely done. **So recipient's SAB test report gives idea about specific HLA antibodies and for virtual cross match with donor's HLA typing.**

Generally, antibodies against donor's HLA A, B and DR is dangerous for hyper acute rejection and such donor should be deferred.

CLINICAL PEARL

4. The advantage of the CDC assay is that it is a functional test involving antibody containing serum and cells. As a crossmatch test it has proved invaluable over the years as a method of avoiding hyperacute rejection due to the presence of HLA-DSA in the recipient.
5. In the context of organ transplantation, however, CDC does have the advantage of only detecting CB (complement binding) antibodies. The rationale for replacing this assay with the solid phase assays was driven primarily by the sensitivity issue and the realization that HLA antibodies positive by the solid phase assays but negative by CDC in some cases were clinically relevant.
6. The Luminex® bead assay is a sensitive method for detection of HLA antibodies and represents the current highpoint in the evolution of HLA antibody detection assays. The additional sensitivity provided by this method has

enabled the detection of HLA antibodies in potential transplant patients which are not detectable by other means, particularly CDC.

7. The development of SAB has enabled the dissection and specificity determination of complex mixtures of HLA antibodies which is not possible with other technique.
8. Although the bead assay represents the most sensitive method for HLA antibody detection one of the main challenges facing clinicians and laboratory scientists is the interpretation of positive results in the context of a negative CDC crossmatch and/or a negative flow crossmatch, and no indication of presensitization by any other screening technique. In such cases other factors need to be assessed like prior history of sensitization, MFI value of detected antibodies and specificity of HLA antibodies. (table 2).
9. Unlike the CDC assay which by definition only detects CB (complement binding) HLA antibodies, the bead assay is designed to detect both CB and NCB (non-complement binding) antibodies. But modified DSA using C1q assay and c3d assays can identify clinically significant antibodies.
10. Non-HLA antibodies are also increasingly being recognised as clinically relevant predictors, and cannot be accounted for utilising SAB method solely with the ever-growing list of HLA alleles, the complete spectrum of unique HLA antigens cannot be fully presented on solid phase assays.
11. The SAB - Luminex® assay has been shown to be susceptible to an artefact known as the prozone phenomenon and can give false negatives with sera high titter anti-HLA antibodies.
12. A similar scenario can arise with the binding of IgM antibodies or other serum factors to the beads and can produce false positive.
13. Drugs such as intravenous immunoglobulin (IVIG) could also interfere with the specific binding of anti-HLA antibodies to the HLA antigens on beads.
14. Another cause for a false negative result is epitope sharing. Different HLA antigens on different beads share mutual antibody binding epitopes leading to the binding of an anti-HLA antibody to more than one bead. This leads to a reduction in the mean fluorescence intensity (MFI) on a single bead.
15. Pre transplant SAB should be done in the patients who have frequent positive HLA cross matches to find out exactly which antibodies are present and giving cross match positive, if its danger antibodies of HLA class DR, HLA A and B , that donor cannot be accepted. But for example, HLA DP antibodies or few other non-danger antibodies with low or borderline MFI value giving CDC cross match positive then transplant can be done after giving induction therapy.
16. **At our centre at the time of transplant,** we check CDC leucocyte crossmatch and Donor specific antibodies by Flow cytometry with turnaround time of 3.5 to 4.5 hours. In case of doubt, we check single antigen bead test by Luminex platform (turnaround time 3 hours) to know specificity of HLA antibodies and virtual cross match with donor's HLA typing report.

Donor crossmatch result	Cross match method	Current (C) or historical (H)	Antibody screening results	Interpretation of immunological risk
Positive T and B lymphocyte	CDC	C	IgG HLA class I DSA	Hyperacute rejection
Positive B lymphocyte	CDC	C	IgG HLA class II DSA	High risk
Positive B lymphocyte	CDC	C	Weak IgG HLA class I DSA	Intermediate risk
Positive T and B lymphocyte	FCXM (CDC neg)	C	IgG HLA class I DSA	Intermediate risk
Positive B lymphocyte	FCXM (CDC neg)	C	IgG HLA class II DSA	Intermediate risk
Positive T and B lymphocyte	CDC	H	IgG HLA class I DSA	High risk
Positive B cell	CDC	H	IgG HLA class II DSA	High risk
Positive B lymphocyte	CDC	H	Weak IgG HLA class I DSA	Intermediate risk
Positive T and B lymphocyte	FCXM (CDC neg)	H	IgG HLA class I DSA	Intermediate risk2
Positive B lymphocyte	FCXM (CDC neg)	H	IgG HLA class II DSA	Intermediate risk2

Table 2: interpretation of various CDC and FCXM cross match results.

To Be continued...

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PRESIDENT

DR JULIUS PUNNEN

Mob: 9980072785

Email: jpunnen@hotmail.com

VICE PRESIDENTS

DR AJITKUMAR V K

Mob: 9895153684

Email: ajitkumarvk@yahoo.com

DR P CHANDRASEKHAR

Mob: 9443047152

Email: chanpad@gmail.com

SECRETARY

DR S RAJAN

Mob: 98412 71353

Email: dr_rajan_icvd@hotmail.com

JOINT SECRETARY

DR RAJAGOPAL S

Mob: 9747606600

Email: srajagovindam@gmail.com

TREASURER

DR JACOB ABRAHAM

Mob: 9847128123

Email: jacabraham1@gmail.com

IMMEDIATE PAST PRESIDENT:

DR V NANDAKUMAR (2019-2023)

Mob: 9843015888

Email: drvnandakumar@gmail.com

PAST PRESIDENTS PERMANENT:

DR JOSE CHACKO PERIAPURAM

Mob: 9847043224

Email: joseperiapuram@hotmail.com

DR K VENUGOPAL

Mob: 9847005444

Email: venugopalknair@gmail.com

PAST PRESIDENTS

DR GEEVAR ZACHARIAH

(2013-2014 and 2014-2015)

Mob: 9846066816

Email: geevarzachariah@gmail.com

DR SHIV K NAIR (2015-2016)

Mob: 9846010220

Email: Shivnairmd@gmail.com

DR K VENUGOPAL (2016-2017)

Mob: 9847005444

Email: venugopalknair@gmail.com

DR JOSE CHACKO PERIAPURAM (2017-2018)

Mob: 9847043224

Email: joseperiapuram@hotmail.com

DR P P MOHANAN (2018-2019)

Mob: 9846076006

Email: drppmohanan@yahoo.com

MEMBERS

DR BASHA KHAN

Mob: 8296825550

Email: bashajalal@gmail.com

DR ADITI SIGHVI

Mob: 9999445511

Email: aditisinghvi4@gmail.com

DR K U NATARAJAN

Mob: 9895057660

Email: kunatarajan@aims.amrita.edu

DR JAMES THOMAS

Mob: 9892797060

Email: thomasdrjames@yahoo.in

DR MANOJ DURAIRAJ

Mob: 9822322072

Email: manojdurairaj@hotmail.com

DR NAGAMALLESH

Mob: 9880135258

Email: nmalesh@yahoo.co.in

DR JAYAGOPAL P B

Mob: 9847023777

Email: jaigopallakshmi@gmail.com

DR PRADEEP KUMAR

Mob:

Email:

DR BAGIRATH

Mob: 9845144830

Email: drbagirath@gmail.com

DR JO JOSEPH

Mob:

Email:

DR TALHA MEERAN

Mob: 9167048815

Email: talha.meeran@gmail.com

DR JABIR ABDULLAKUTTY

Mob: 9447011773

Email: drjabi@yahoo.co.in