COMPARISON OF HLA MISMATCH SCORING AND THE PRE-TRANSPLANT SERUM DONOR SPECIFIC ANTIBODY (DSA) LEVELS WITH HISTOMORPHOLOGY TO PREDICT THE ANTIBODY-MEDIATED REJECTION AS WELL AS THE SEVERITY OF REJECTION

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ABSTRACT

BACKGROUND
Immunologic studies such as Human Leukocyte Antigen (HLA) typing and measurement of the Donor Specific Antibody (DSA) titre are more helpful to assess the long-term success of renal transplantation and in a superior graft survival. Amount of HLA mismatching and the immune response against the graft determine the success of renal transplantation and graft versus host disease. Rejection can be a humoral or T-cell mediated, but the humoral rejection is more important, as it has a very few diagnostic morphological features in the biopsy and these cases are resistant to anti-rejection therapy with a worse prognosis.

MATERIALS AND METHODS
Retrospective study conducted in the Department of Pathology, Sri Venkateshwaraa Medical College Hospital and Research Centre, Puducherry, on the renal allograft rejection biopsies/cases between January 2013 and December 2015; 50 rejection cases/biopsies were analysed in respect to the clinical, histological and their serological/immunological status (HLA typing and serum DSA level).

RESULTS
In this study, significant association was found between the antibody-mediated rejection cases as well as their severity with the level of HLA mismatch scoring (> 50 percent). Pre-transplant DSA levels were found to have no value in predicting the antibody-mediated rejection.

CONCLUSION
It is very difficult to evaluate the clinical outcome as well as the graft survival by measurement of pre-transplant Donor Specific Antibody (DSA) level alone. Pre-transplant DSA level does not predict the possibility of humoral rejection later.

KEYWORDS
Banff, HLA, DSA, Humoral Rejection, C4d.

BACKGROUND
Transplantation of an allogeneic kidney stimulates the recipient's immune system to mount a response, which in the absence of substantial immunosuppression rejects the allograft. It also represents a manifestation of the immune systems function to distinguish self from non-self and reflects recognition of foreign antigens including the products of the Major Histocompatibility Complex (MHC) and minor Histocompatibility genes.[1-4] It may be either MHC class I antigens (HLA-A and HLA-B) or class II antigens (HLA-DR, DP, DQ) and ABO blood group antigens.[5] Panel Reactive Antibody (PRA or DSA) levels are routinely performed before kidney transplantation. It is a way of measuring the anti-HLA antibodies[6,7,8] in the serum. A person's PRA can be anywhere

from 0% to 99%. PRA represents the percent of the anti-HLA antibodies in recipient serum against a set panel of HLA antigens[6,7,8] and it can identify the risk of hyperacute or vascular rejection.

Large multicentric studies[9] have shown that there is a significant beneficial influence of HLA matching/typing on the graft outcome. It also clearly showed the benefit of haplotype matching; zero, one and two haplotype matched primary renal grafts had three year graft survival[9] of 74%, 80% and 85% respectively. Acute humoral rejection may be associated with appearance of donor specific antibodies that can be detected by flow cytometric assay or ELISA method. DSA levels are not correlated with histological features of humoral rejection.

In view of these observations, Banff (1997) classification was revised in 2003[10] incorporating morphological criteria with supported immunological status. Morphologically, the changes are classified[10] into,
1. Borderline.
2. Antibody-mediated rejection.
3. Acute/active cellular rejection.
4. Chronic/sclerosing allograft nephropathy.

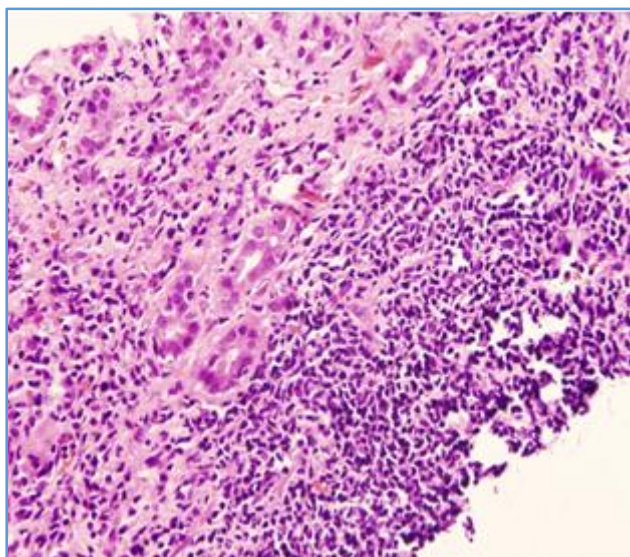Humoral or antibody-mediated rejection manifest as any one of the following three types[10,11]

I – acute tubular injury.

II – neutrophils in peritubular capillaries.

III – fibrinoid necrosis of arteries.

Biopsies that meet the criteria for both AHR and ACR type I or II are considered to have both forms of rejection. C4d positive biopsies without acute inflammation, but with chronic injury (GBM duplication, arteriopathy, interstitial fibrosis and tubular atrophy) are considered to be chronic antibody-mediated rejection.[10] Biopsies with C4d and no pathology may be a manifestation of accommodation.
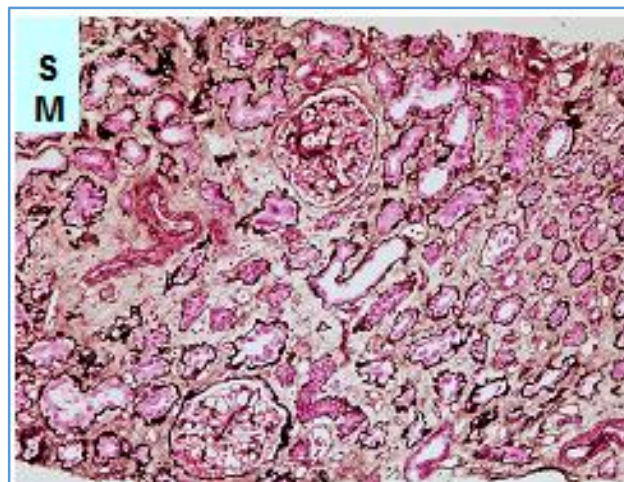
MATERIALS AND METHODS

50 rejection cases received between January 2014 and December 2015 were collected from the RG Urology Hospital, Chennai and analysed retrospectively with their pre-transplant workup. Biopsy workup with immunohistochemical study was done at Sri Venkateshwaraa Medical College Hospital and Research Centre, Puducherry. All the biopsies were indicated (only rejection cases by clinically with adequate tissue) and performed for the diagnosis of graft dysfunction. Pre-transplant workup done from the Apollo Main Hospitals, Chennai and their HLA typing status, pre-transplant DSA/PRA levels were collected from their serology/immunology section.

Histological sections are studied by staining with H and E, PAS (Periodic acid-Schiff) and Silver (SM) stains. All the rejection cases were confirmed and subclassified by using Banff 2003 criteria.[12-14] (Figure 1 to 4). C4d, a complement product of classical pathway[12] (triggered by anti-donor antibody) is used as a durable marker for predicting antibody-mediated rejection.[15,16] Immunostaining for C4d and CD34 was done by Labelled Streptavidin Biotin immunoperoxidase technique (LSAB) using monoclonal antibody to C4d and CD34 on serial step sections (Figure 3 and 4).
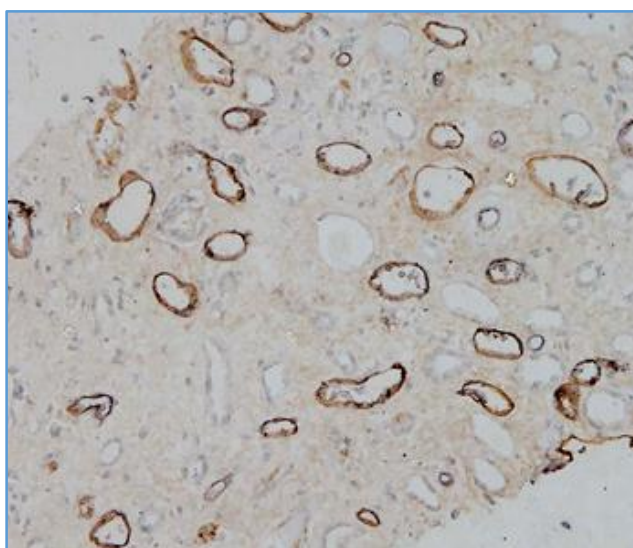

Figure 2. Tubular Atrophy and Interstitial Fibrosis
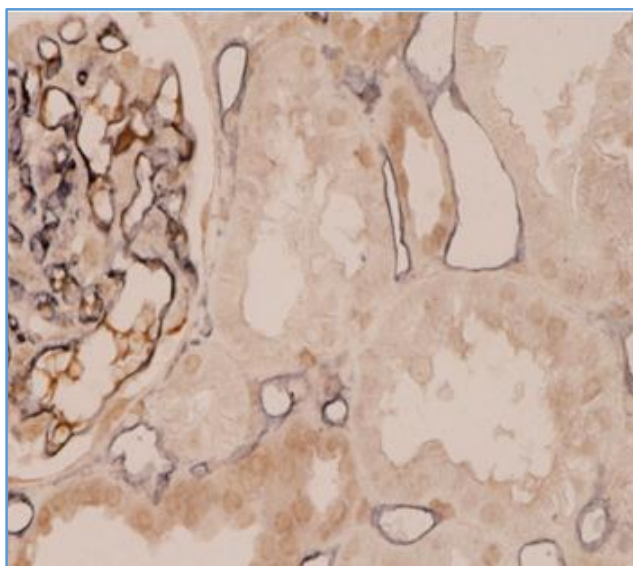
Figure 3. Positive Internal Control with C4d Negative PTC

Figure 1. Tubulitis and Interstitial Inflammation

Figure 4. Diffuse C4d Positive PTC

Lymphocytotoxicity Test (by serology) for HLA Typing[17-21]

The peripheral blood lymphocytes were generally used for HLA testing, because of easy availability and separation. Lymphocyte suspension for cytotoxicity test should be obtained from fresh blood samples, although 24 hrs. old blood can also yield viable cells if processed carefully. Peripheral Blood Lymphocytes (PBLs) were separated on the basis of density gradient centrifugation. The density gradient used was Lymphoprep, which has a specific density of 1.077 - 1.080 at 20°C.

Procedure for HLA Typing[17-21]

HLA class antigen (A, B, C) and cross-match testing can be performed by either: Fresh peripheral blood lymphocytes separated from peripheral blood lymphocytes using nylon wool. The suspension should be adjusted to a final concentration of 2.0 - 2.5 X 10^{6} cells/mL.

- The lymphocyte suspension should be > 90% pure and free of contaminants. For class I (HLA - A, B, C) typing, the cell concentration should be adjusted to 2.0 - 2.5 x 10^{6} cells/mL.
- Trays should be thawed and brought to room temperature before use. Checked for empty wells and mark them.
- Added 1 μL of lymphocyte cell suspension of each well of the test tray using a Hamilton 50 μL repeating microsyringe. Care should be taken to ensure proper mixing of the sera and cells.
- Incubated the trays at room temperature (20 - 25°C) for 30 mins.
- Added 5 μL of rabbit complement (pre-screened) using a 250 μL microsyringe or a multi-dispenser. Made sure that it gets mixed properly.
- Incubated the trays at room temperature (20 - 25°C) for 1 hr.
- Added 5 μL of 5% eosin dye (water soluble). Two minutes later, add 5 μL of formalin (pH 7.2 - 7.4) to each well. Both these reagents were added using a multiple dispenser attached to a jet pipette system (Robbins Scientific Corporation, USA).
- Minimum one hour for the lymphocytes to settle down before reading the results.
- Microscopic evaluation.[17-21]
- Evaluation of reading the trays is done by using an inverted/phase contrast microscope. Live cells that exclude dye are small and retractile. Dead lymphocytes that uptake dyes are larger and stained dark red.
- Scoring was done by estimating the percentage of cell lyses.
- The following International scoring system is generally employed, which is also convenient. Typing trays are scored on 'subjective scales' taking into consideration the amount of 'background' (dead cells) in the negative control well.

Score Interpretation Dead Cells (%)

1	Negative	0 - 15
2	Doubtful Negative	16 - 25
4	Weak Positive	26 - 50
6	Positive	51 - 80
8	Strongly Positive	81 - 100
0	Not readable	

Estimation of Donor Specific Antibodies[6-8] (DSA or PRA)

HLA antibodies can be acquired through alloimmunisation and it leads to production of HLA antibodies in approximately 33% of exposed individuals. It is estimated by ELISA method and the patient's serum is added to microwell and they are already bound with HLA antigen reacts with antibody present in the serum. Unbound antibodies are then washed away and anti-IgG antibodies, substrate PNPP were added. After 30 minutes of incubation period, the reaction is stopped by a sodium hydroxide solution. The optical density of the colour that develops is measured in spectrophotometer.

Test results showing OD values equal to or greater than cut-off value are regarded as being positive result.

Calculate percentage of PRA[6-8]

$$PRA\ (\%) = \frac{\#\ of\ positive\ result}{\#\ of\ wells\ containing\ HLA\ class\ I\ antigen} \times 100$$

Patients are then categorised into 3 groups depending on the reactivity pattern with panel members.

0 - 11%	Negative.
11 - 50%	Moderately sensitised.
75%	Highly sensitised.

Data Analysis

Humoral/AMR rejection cases (C4d deposition in the peritubular capillaries) and the cellular rejection biopsies as per the BANFF 2003 criteria were correlated with their HLA mismatch scoring, pre-transplant PRA levels. Fisher Exact 't' Test (Parametric Test) was done to compare the C4d deposition in different grades of rejection with HLA status/PRA levels.

RESULTS

In this study 43 cases were male, 7 female and their age groups were ranging between 17 to 51 yrs.; 39 patients (78%) had live related donor, 10 had live unrelated and 1 had cadaver donor transplantation. End stage glomerular diseases, diabetic nephropathy (55 to 65%) were the common indication for the transplantation and the other causes were chronic interstitial nephritis and adult polycystic kidney disease. Graft dysfunction was manifested from the first week of post-transplant till the 5 yrs.

PRA Level

Significant PRA levels (> 10%) were found in 7 patients with the mean value of 21.29 ± 9.96. Among these 3 (43 percent) patients were found to have chronic allograft nephropathy, 1 (14 percent) had Acute Cellular Rejection, 1 (14 percent) had combined acute cellular and chronic rejection and 2 (29 percent) patients were found to have no rejection in our study.

HLA Mismatch Scoring (Table 1)

In this study cases were categorised into 6, 5, 4, 3, 2, 1, 0 mismatches on the basis of their HLA disparity. HLA mismatch '3' was found in 30 cases (60 percent). HLA mismatch '4' was found in 3 cases (6 percent). Mismatch '5' was found in 10 cases (20 percent). HLA mismatch '6' or totally unmatched transplants were found in 7 cases (14 percent). None of them found to have complete matching (0) or 1, 2 mismatch.

Based on the HLA mismatch, cases were broadly classified into two groups.
1. Less than 50% (< 50%) HLA mismatching (HLA mismatch 3, 2, 1, 0).
2. More than 50% (> 50%) HLA mismatching (HLA mismatch 6, 5, 4).

In this study, < 50% HLA mismatches were found in 30 cases (60 percent). Rest of the 20 cases (40 percent) were shown > 50% HLA mismatch.

HLA Mismatch Scoring	No. of Cases (n = 50) (%)
Less than 50% mismatch	30 (60)
More than 50% mismatch	20 (40)
Table 1	

Comparison of C4d positive cases with their histological Banff ACR grades (Table 2).

Banff (ACR) Grade	C4d Positive (n=12) (%) (p = 0.014)	C4d Negative (n=38) (%) (p = 0.014)
0	2 (17)	21 (55)
Borderline	0 (0)	4 (11)
Ia	1 (8)	0 (0)
Ib	1 (8)	0 (0)
IIa	3 (25)	6 (16)
IIb	5 (42)	7 (18)
Table 2		

Comparison of C4d positive cases with their HLA mismatch scoring status: Association between these two variables was statistically significant (Table 3).

HLA Mismatch Score	C4d Positive (n=12) (%) (p = 0.007)	C4d Negative (n=38) (%) (p = 0.007)
< 50% (0, 1, 2, 3)	3 (25)	27 (71)
> 50% (4, 5, 6)	9 (75)	11 (29)
Table 3		

DISCUSSION

Statistically significant association (p < 0.05) was found between the grade of rejection and the C4d positivism. Higher the grade of acute cellular rejection (Banff criteria), more the chances of getting C4d deposition in PTC. C4d positive capillaries were found unevenly distributed among the various grades of chronic allograft nephropathy. The larger numbers of cases were found in grade I (5 cases, 42 percent) and grade 0 (4 cases, 33 percent). Only one case showed C4d deposition grade III CAN.

Comparison of C4d positive cases with PRA levels: Significant PRA levels (> 10 percent) were found in 7 patients with the mean value of 21.29 ± 9.96. None of them found to have C4d positive capillaries. C4d positive patients with the post-transplant DSA level of more than 30 percent found to have highly resistant to standard anti-rejection therapy and a greater rate of degree of graft loss.[22,23] In this study, pre-transplant PRA level was found to have no value in predicting

either the antibody-mediated rejection or C4d deposition on peritubular capillaries. Statistically significant correlation (p < 0.05) was found between the higher degree (> 50 percent) of HLA mismatches and the chances of getting humoral or antibody-mediated rejection as well as the higher grade of rejection.

CONCLUSION

It is very difficult to evaluate the clinical outcome as well as the graft survival by measurement of pre-transplant Donor Specific Antibody (DSA) level alone. There are chances of shedding antibodies from the graft endothelial cell surface, which results in detection of DSA by commonly available techniques difficult. So the combination of post-transplant DSA with HLA mismatch typing can be used as better graft monitoring investigation. Pre-transplant DSA level does not predict the possibility of upcoming humoral graft rejection.

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