ORIGINAL ARTICLE

A comparison of positivity using routine incubation, extended incubation and antihuman globulin in the complement dependent cytotoxicity (CDC) assay

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ABSTRACT

Objectives: This study quantified and compared positivity obtained on complement dependent cytotoxicity (CDC) using routine incubation, extended incubation and anti-human globulin.

Methods: This was a retrospective study which included results from all samples processed for CDC as part of pre-transplant screening in our laboratory in 2013. Samples were processed in parallel for routine incubation CDC, extended incubation CDC and anti-human globulin CDC techniques. Positivity in terms of percentage dead cells was recorded for each technique. All samples that showed positive results (>/=10% dead cells) by at least one method were included in statistical analysis to compare degree of positivity. Negative samples and those that failed validation controls by any technique were excluded. Results of extended incubation CDC were analysed in 131 samples, routine incubation CDC in 103 and anti-human globulin CDC in 111. Results were recorded as percentage dead cells and these values were compared between techniques using the paired t test.

Results: Comparison of reactivity of extended incubation CDC and anti-human globulin CDC with routine incubation CDC, showed a highly significant difference ($p<0.0001$ and 0.003 respectively) with a mean increase in positivity of 7% over routine incubation with both extended incubation CDC and anti-human globulin CDC. Comparison of extended incubation CDC to the anti-human globulin CDC showed no significant difference $p=0.19$).
Routine incubation missed positivity in 30% of the positive samples tested.

Conclusions: Anti-human globulin and extended incubation enhance the positivity of CDC by approximately 7%. However, neither anti-human globulin CDC nor extended incubation CDC showed any significant increase in positivity over each other.

Key words: anti-human globulin, complement dependent cytotoxicity, crossmatch, extended incubation, human leukocyte antigens.

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INTRODUCTION

The complement dependent cytotoxicity (CDC) assay since the late 1960s has been a mainstay of pre-transplant testing for anti - HLA antibodies (1,2) Many laboratories continue to report on the CDC with routine incubation timings, while others use various enhancement techniques to improve its sensitivity, two favoured ones being the use of extended incubation CDC and antihuman globulin CDC (2). Laboratories worldwide use either of these methods to enhance the CDC. There are few studies that have actually measured the degree to which these methods enhance the positivity obtained on routine incubation CDC.

Our study estimated the increase in positivity obtained using each of these enhancement methods over the standard CDC, and also compared them with each other to find if either enhances positivity of the other.

MATERIALS AND METHODS

This was a retrospective study that collated data from all cross-matches performed in our laboratory in 2013, as part of pre-transplant screening for living donor renal transplants. All samples were processed for donor recipient crossmatch using routine incubation CDC, extended incubation CDC and anti-human globulin CDC. The cross-match procedure was as follows:

Primary sample and processing

Blood for lymphocyte separation was collected from the donor (who was instructed to arrive in the morning in a fasting state) in Acid Citrate Dextrose 1 and processed immediately. Lymphocytes were separated into 5% McCoy's by density gradient centrifugation using Lymphoprep (Axis-Shield PoC AS, Norway). Contaminating erythrocytes were lysed using tris ammonium chloride solution. Platelets were aggregated using thrombin and removed by sedimentation. Viability was checked after the primary separation and should be at least 95%. In case the quality of separation was unsatisfactory, further processing using Percoll (Sigma Aldrich, USA) was undertaken. Lymphocytes were adjusted to a concentration of 1.5-2.5 million/ml for the crossmatch. Ten ml of blood collected from the patient in a clot enhancer tube and allowed to clot at room temperature following which serum was separated and used for the cross-match.

Cross-match procedure

One microliter of cell suspension was dotted onto Terasaki trays. One microliter each of serum in neat (N), N/2 and N/4 dilutions, and rabbit complement (One Lambda, USA; lot #030 was used during the entire study period) were added. For routine incubation timings, incubation periods of half an hour following addition of serum and one hour following complement were followed. These incubation periods were doubled for extended incubation cross-matches.

For the anti-human globulin cross-match, cells and serum wee incubated for forty-five minutes followed by three manual washes on the tray using 5% McCoy solution. Subsequently the tray was re-oiled followed by addition of anti-human globulin (goat IgG anti human kappa, One Lambda, USA; lot #007 was used for the entire period of the study) in dilutions of N/32, N/64 and N/128. Undiluted complement was added followed by ninety minutes of incubation. Subsequently vital dye (aqueous eosin) was added and the reaction was fixed by adding 5% formalin after 5 minutes and read. The trays were read on an inverted phase contrast microscope and the percentage dead cells in each dilution were recorded.

All incubations occurred at controlled room temperature (22 to 26 degrees Celsius). Appropriate positive and negative controls were dotted in parallel in each tray. In the negative control wells, serum from a non-sensitised male was added in similar dilutions as the corresponding test wells. In addition, to control for background cell death, in one well, phosphate buffered saline was added to the cell suspension instead of serum. Each sample was processed by the three techniques in parallel and read by the same individual. Reactivity was measured in terms of percentage of dead cells in test wells over that in the negative controls. The CDC is a subjective test. Therefore, the test was read by a staff member with at least five years of experience in reading CDC crossmatches.

For this study, the maximal reactivity obtained by each method on a sample was recorded. Any sample that showed positivity of 10% or more on at least one technique was included for analysis. A cut off of 10% was used as this was considered the minimal degree of positivity that was consistently interpreted as positive by different observers. Tests that had failed controls or where background cell death exceeded 10% were excluded from comparison for that technique. Also, where patient samples were limited, routine incubation CDC was not performed. Towards the end of the year, in recognition of the lack of sensitivity, routine incubation CDC was discontinued. A paired t test was used to determine any significant difference in reactivity between the two methods.

RESULTS

354 CDC crossmatches were performed during the study period. 131 samples (37%) were positive by at least one method and were included in our analysis. All of these had been processed by the extended incubation CDC protocol.

In addition, 103 of these samples had been processed using routine incubation CDC, and 111 using anti-human globulin CDC (Figure 1).

Only 72 out of 103 (70%) were positive on routine incubation CDC. 14 samples were positive on extended incubation CDC but negative on anti-human globulin CDC, with positivity on the former ranging from 10-15%. Seven of these were also positive on routine incubation. Nine samples were likewise positive on anti-human globulin CDC but negative on extended incubation CDC, with positivity with anti-human globulin ranging from 10-15% in all but one sample. This sample alone showed strong positivity of 60% with anti-human globulin which was not detected by extended incubation. None of the samples that were positive with anti-human globulin CDC but not with extended incubation. CDC were positive on routine incubation (Tables1 and 2, and Figure 2).

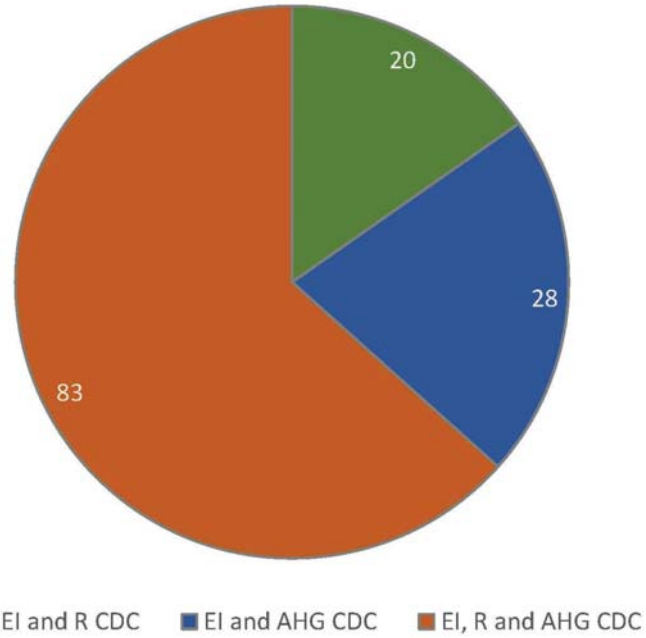

Figure 1. Pie chart showing the number of samples included under each method. (EI=extended incubation; R=routine incubation; AHG=anti-human globulin).

Table 1. Distribution of reactivity among samples processed by each technique.

Reactivity (% dead cells)	Routine incubation Number of samples (%)	Extended incubation Number of samples (%)	Anti-human globulin Number of samples (%)
</=20%	79 (77)	90 (69)	76 (69)
21-50%	3 (3)	9 (7)	8 (7)
51-80%	5 (5)	9 (7)	9 (8)
81-100%	16 (15)	23 (17)	18 (16)
Total	**103 (100)**	**131 (100)**	**111 (100)**

Table 2. Number of positive samples missed by each technique.

Technique	Number of samples processed	Number of samples where antibodies were missed (%)
Routine incubation CDC	103	31 (30)
EI CDC	131	9 (7)
AHG CDC	111	14 (13)

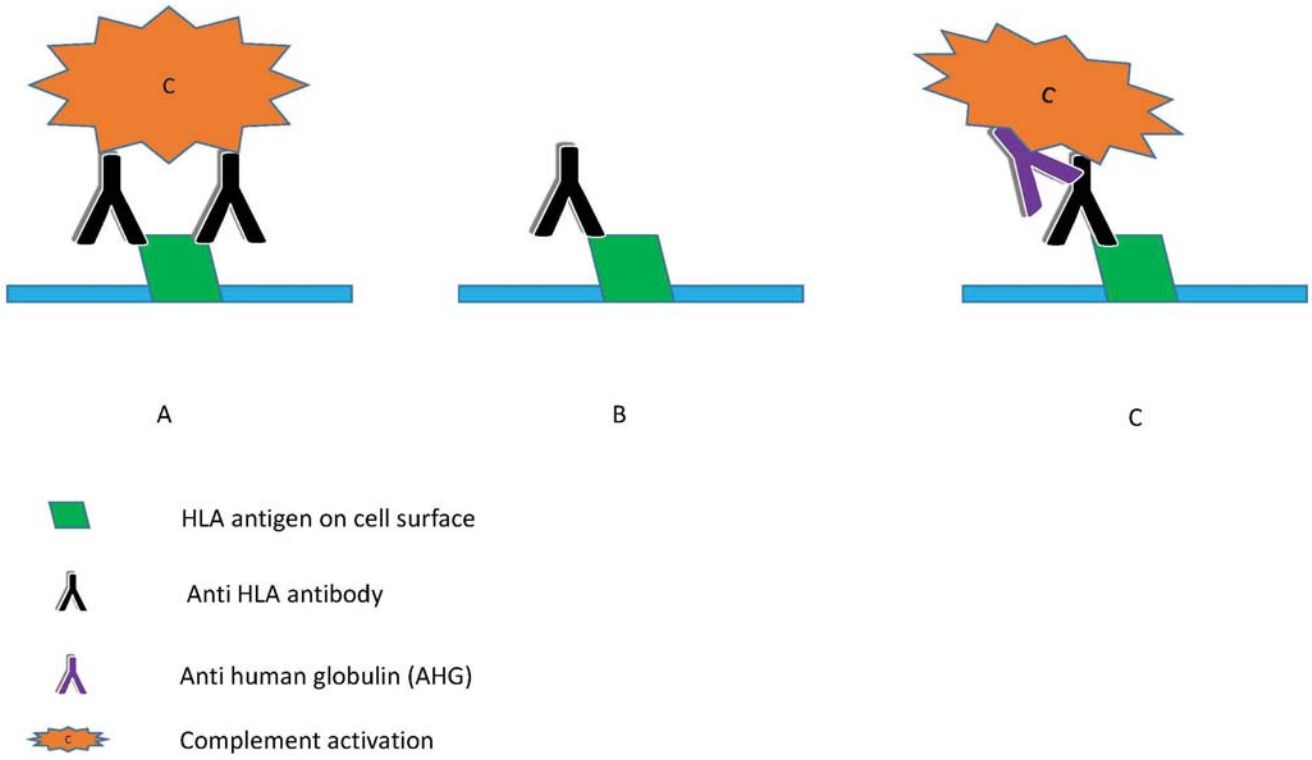

Figure 2. Diagram showing the mechanism of complement fixation by IgG and the CYNAP phenomenon. A) IgG antibodies bound to different epitopes on the same molecule together activate complement. B) IgG antibody binding to a single epitope on a molecule cannot activate complement on its own. C) Antihuman globulin binds to the primary antibody and associates with it to activate complement.

Comparison of reactivity of extended incubation CDC with routine incubation CDC, showed a highly significant difference ($p<0.0001$; $t = 4.81$) with a mean increase of 7% (standard error 1.49). Comparison of anti-human globulin CDC with routine incubation CDC also showed a significant difference ($p=0.003$; $t = 3.03$) with a mean increase in reactivity of 7% (standard error 2.3). Comparison of extended incubation CDC to the anti-human globulin CDC showed no significant difference (two tailed $p=0.19$; $t=-1.33$) with a mean of differences equal to 2.3% (standard error 1.76) (Figure 3).

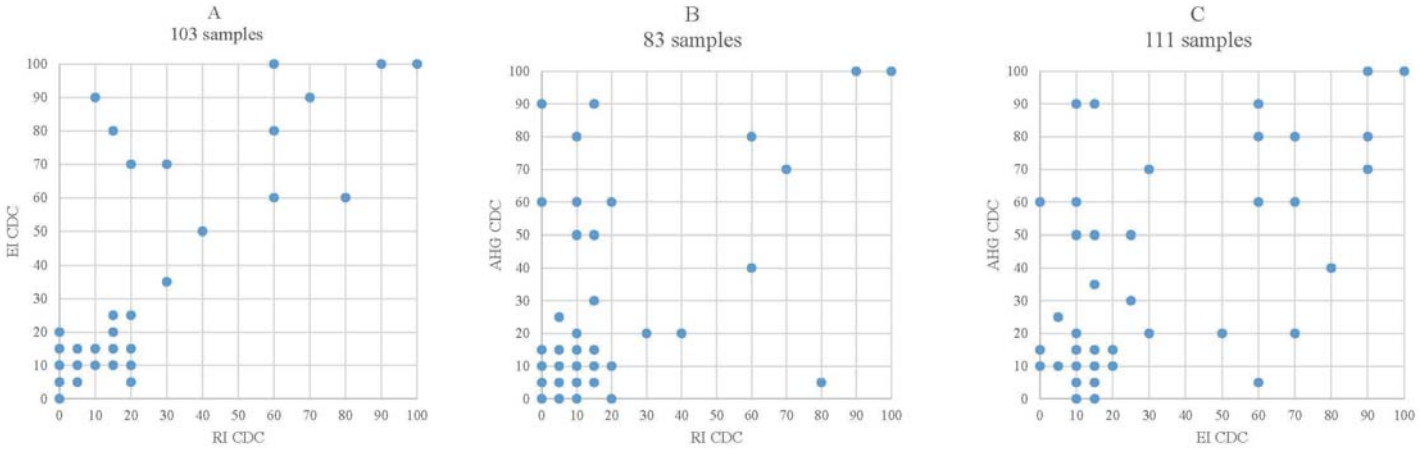

Figure 3. Scatter plots comparing positivity exhibited by samples with various techniques. A) routine incubation CDC (x axis) versus extended incubation CDC (y axis). B) Routine incubation CDC (x axis) versus anti-human globulin CDC. C) Extended incubation CDC (x axis) versus anti-human globulin CDC (y axis). (RI=routine incubation; EI=extended incubation).

DISCUSSION

The CDC crossmatch has been a decisive test in pre-transplant compatibility testing ever since its high prediction for early acute rejection in renal transplants was brought to attention by Patel and Terasaki (1). Among the various platforms available in the market today, CDC alone has shown consistent and strong correlations with post- transplant outcomes.(1,3-5) However, CDC is often criticized for its lack of sensitivity as compared to other platforms. Modifications aimed at enhancing antibody detection of CDC almost immediately followed its inception. These include the anti-human globulin technique, and extended incubation (6,7).

In our study, as expected, both enhancement methods produced a significant and comparable increase in positivity over routine incubation CDC with a mean increase in reactivity of 7% for both extended incubation CDC and anti-human globulin CDC over routine incubation CDC. However, there was no statistically significant enhancement in positivity observed with either of the enhancement techniques as compared to the other, even though anti-human globulin CDC is generally declared to be more sensitive.

The use of anti-human globulin enhances the detection of weak or low titred antibodies as well as non-complement fixing antibodies, including cytotoxicity negative adsorption positive (CYNAP) antibodies (8-11). Complement activation requires binding of complement by at least two nearby Fc segments, requiring that they be within a distance of 14 angstroms on the cell surface (9,10). IgM antibody by virtue of its large pentameric structure has five Fc fragments per molecule and will fulfil this requirement. IgG, with its monomeric structure (with only one Fc fragment per molecule) will only fulfil this requirement where there are multiple antibodies clustered on a single HLA molecule. Consequently, where there is IgG with only a single specificity, complement activation may not occur. Anti-human globulin circumvents this by binding to the primary antibody, allowing participation of its own Fc fragment with that of the primary antibody in complement activation, thereby allowing detection on CDC (10,11) (Figure 3). Notably, sera that have multiple specificities binding to the same antigen, based on this principle *are not enhanced by using anti-human globulin as they already effectively activate complement* (9). This probably explains why our study did not show any overall enhancement of positivity when using anti-human globulin CDC as compared to extended incubation CDC. Extended incubation on the other hand increases the time period allowed for antigen-antibody interaction and complement fixation. This probably improves detection of weak antibodies but still does not allow for detection of non-complement fixing antibodies (8-10).

Cross *et al.* first introduced the anti-human globulin technique, and observed that it had higher sensitivity and enhanced the reaction as compared to extended incubation CDC. They also noted that all patients who were transplanted across a positive anti-human globulin CDC but negative extended incubation CDC developed accelerated or acute rejection within two months post-transplant, whereas those who were transplanted across a positive extended incubation crossmatch but negative anti-human globulin CDC had neither hyper-acute or accelerated rejection (7). Interestingly, all the sera in their study that were solely positive on extended incubation were also auto -reactive. This would explain absence of rejection in these cases, as auto-antibodies are generally considered harmless and not a contra-indication for transplant (2,12). The clinical significance of CYNAP antibodies is debated. It is suggested that these antibodies pose some risk but are not an absolute contra-indication for transplant (2).

Cross and others have demonstrated enhancement of the reaction, detection of antibody in higher dilutions, and enhanced binding of complement when using anti-human globulin even when direct CDC was positive (6,10). However, methodological differences are known to cause wide variation in the sensitivity of CDC crossmatches and others have demonstrated achievement of sensitivity comparable to anti-human globulin CDC merely by increasing serum and complement incubation timings (2,13).

As in Cross's study, certain sera in our study showed positivity on only one of the enhancement techniques. Missed positivity was more frequent with anti-human globulin. Cross *et al.* showed that crossmatches that were positive on extended incubation alone in their study became negative in two cases on introducing a wash step (7). This suggests that weakly bound antibodies may be lost during the wash steps in the anti-human globulin CDC technique. Our finding that at least some of these samples that were negative on anti-human globulin CDC were positive on routine incubation as well as extended incubation further implicates the wash step in the loss of antibody. On the other hand, positivity that was evident on anti-human globulin CDC but not on extended incubation CDC may have been due to CYNAP antibodies, though this could not be proven.

The antibodies picked up by either technique alone in our study were weak, with one exception. This case showed strong positivity with anti-human globulin which was not evident on extended incubation CDC. Moreover there was an apparent prozone effect with positivity increasing from 5-10% in neat serum to 60% with N/4 dilution. The positivity was confirmed on the Luminex crossmatch assay using donor lysate which showed class I positivity with a median fluorescent intensity median fluorescent intensity (MFI) of 3928.

CONCLUSIONS

As has been previously reported, anti-human globulin CDC and extended incubation CDC enhance the positivity of the routine incubation CDC. The increase in positivity using both enhancement methods was approximately 7%. Positivity obtained on anti-human globulin CDC and extended incubation CDC was comparable. i.e. anti-human globulin CDC was not found to show any significant enhancement of positivity over that obtained on extended incubation CDC.

We also found that both anti-human globulin CDC as well as extended incubation CDC used in isolation could potentially miss some positive samples. As none of our positive patients were transplanted without undergoing desensitisation, the clinical implications of this remain uncertain. Towards the end of the year, our laboratory discontinued the routine incubation CDC in view of its lack of sensitivity. We continue to perform extended incubation CDC and anti-human globulin CDC on all samples in parallel.

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