Complement-Dependent Cytotoxicity (CDC) to Detect Anti-HLA Antibodies: Old but Gold

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Background: The criterion (gold) standard to detect anti-human leukocyte antigen (HLA) antibodies is the complement-dependent cytotoxicity (CDC) assay. Recently, more sensitive methods have been used for the same purpose. **Methods:** This study analyzed 70 serum samples of patients with end-stage renal disease using CDC, CDC with the addition of anti-human globulin (CDC-AHG), CDC with the addition of dithiothreitol (CDC-DTT), and the recent solid-phase immunoassay (SPI; Lab-screen PRA) to detect anti-HLA antibodies. **Results:** Mean percent panel reactive anti-

bodies (PRA) detected by SPI was 37.5% (± 34.2) higher than the values detected by the other methods. Comparative analyses revealed significant difference between CDC and CDC-AHG, and between CDC and SPI ($P < 0.0001$), but not between CDC-AHG and SPI ($P = 0.8026$). **Conclusion:** Although the CDC-AHG method is “old,” its performance to detect anti-HLA antibodies in the samples analyzed was comparable to the SPI in the evaluation of percent class I PRA. J. Clin. Lab. Anal. 28:275–280, 2014. © 2014 Wiley Periodicals, Inc.

Key words: alloantibodies; HLA; CDC; solid-phase immunoassay; transplantation; end-stage renal disease; panel reactive antibodies

INTRODUCTION

Kidney transplant is the best option for the treatment of end-stage renal disease (1). Anti-human leukocyte antigen (HLA) antibodies in the serum of patients who should receive organ transplants are a sign of an important risk factor (2). The method to detect anti-HLA antibodies has historically been the complement-dependent cytotoxicity (CDC) assay, described by Terasaki and McClelland in 1964 (3). Later, CDC with the addition of anti-human globulin (CDC-AHG) or dithiothreitol (CDC-DTT) was developed to improve the classical CDC assay. The addition of AHG enhances the amplification of the cytotoxicity reaction promoted by complement-fixing antibodies found in low titers and increases CDC sensitivity (4). Also, serum adequately treated with DTT improves the detection of antibodies to immunoglobulin G (IgG) and immunoglobulin M (IgM) isotypes (5). The importance of such improved detection lies on the fact that IgG isotype antibodies are deleterious for transplants and lead to hyperacute rejection (6, 7) and IgM isotype antibodies may be associated with the production of autoantibodies, in which case the transplant is not contraindicated (8).

Recently, more sensitive assays to detect anti-HLA antibodies, such as enzyme-linked immunosorbent assays and flow cytometry based assays (9) have contributed to an increase in the number of patients in the waiting lists of organ transplantation, although the clinical relevance of these antibodies remains unclear (2).

Donor-specific antibodies (DSA) detected by cross-match testing using CDC may suggest a contraindication for the transplant (2), so that hyperacute rejection and immediate loss of the graft may be avoided (6, 7). However, DSA detected exclusively by means of more sensitive

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assays should be regarded as an additional risk factor and not necessarily a contraindication to transplant success (2, 10, 11).

Although more sensitive assays have been widely used, CDC remains an alternative to define the level of patient sensitization (12), in crossmatching with a specific donor to prevent hyperacute rejection (13, 14) and detect antibody isotypes (15).

This study evaluated the performance of the CDC assay in comparison with a more recent technology, a solid-phase immunoassay (SPI) using the commercial kit LSIPRA[®] (One Lambda, Inc., Canoga Park, CA) to evaluate percent class I panel reactive antibodies (PRA) in samples of sera of patients waiting for a kidney transplant.

MATERIALS AND METHODS

Study Subjects

Sera of patients with end-stage renal disease

The sera analyzed in this study were collected from patients with end-stage renal disease in a single hemodialysis center in the city of Maringá, state of Paraná, Southern Brazil. All patients enrolled in the center participated in this study ($n = 70$).

PRA detected by CDC

The sera were analyzed using a panel of mononucleated cells isolated from 33 healthy donors. All serum samples were tested with CDC, CDC-AHG, and CDC-DTT. Results were described as percentages; percent PRA was negative when equal to zero (0%), and positive when greater than zero.

Serum treatment with DTT

For CDC-DTT assays, patient sera had to be previously treated with a DTT solution (Ultra Pure[™], Invitrogen[™], Inc., Carlsbad, CA) at a final concentration of 50 mM. Serum and DTT were incubated for 30 min at 37°C and agitated every 10 min. After treatment, sera were placed in a (Terasaki plate, One Lambda, Inc., Canoga Park, California, USA) for the CDC assay.

T-cell panel

For the cell panel, 33 healthy individuals of known HLA typing for A and B locus antigens underwent collection of 8 ml of peripheral blood in a container with acid citrate dextrose (Vacutainer[®] ACD, Becton Dickinson Diagnostic System, Buenos Aires, Argentina) (Table 1). Cells were separated using immunomagnetic

TABLE 1. HLA Specificities Used for the Cell Panel in the CDC Assay

A locus	Frequency	B locus	Frequency	B locus	Frequency
A1	4	B7	9	B52	2
A2	14	B8	3	B53	1
A3	6	B13	1	B55	1
A11	2	B14	3	B57	1
A23	1	B15	4	B58	3
A24	9	B18	6	B60	1
A26	2	B27	2	B63	1
A28	2	B35	5	B70	1
A29	4	B37	1		
A30	6	B40	7		
A31	1	B41	1		
A32	2	B44	5		
A33	1	B48	1		
A68	2	B50	1		
A74	2	B51	3		

CDC, complement-dependent cytotoxicity assay; HLA, human leukocyte antigen.

beads (Fluorobeads[®] -T, One Lambda, Inc.) according to the manufacturer's instructions.

Assays

CDC assay

Initially, 1 μ l of cells at a concentration of 3×10^6 cells/ml were placed onto the Terasaki plates where patient sera had been previously distributed. After incubation at 22°C for 30 min, 5 μ l of rabbit complement (Imunodiagnóstica—Produtos e Serviços em Imunologia Ltda, Curitiba, Brazil) was added, followed by incubation for another 90 min. Immediately after that, the complement was removed from the plate by flickering and 5 μ l of a stain-quench agent (FluoroQuench[™], One Lambda, Inc.) was added. The reaction was visualized under inverted fluorescence microscopy (IBE 2,000, Applied Biosystems, Foster City, CA) and a 10 $\times$ objective lens.

Results were described using scores from 1 to 8. These scores were based on the International Histocompatibility Workshop described by Dyer and Middleton (16). The reaction was positive when scores were equal to or greater than 2.

CDC-AHG assay

After incubation of sera and cells, as in the CDC assay described above, samples were rinsed three times, and 15 μ l of phosphate-buffered saline was added to each well (each rinse, 10-min incubation). After plate flickering, 1 μ l AHG (Goat IgG Anti-Human Kappa, One Lambda, Inc.) and, 1 min later, 5 μ l of rabbit complement (Imunodiagnóstica—Produtos e Serviços em Imunologia Ltda)

TABLE 2. HLA Specificities Used for the Cell Panel in the SPI Test (LS1PRA, batch #10)

A locus	Frequency	B locus	Frequency	B locus	Frequency
A1	9	B7	3	B57	4
A2	17	B8	3	B58	3
A3	6	B13	3	B59	2
A11	10	B18	3	B60	3
A23	5	B27	3	B61	3
A24	6	B35	3	B62	4
A25	3	B37	2	B63	3
A26	5	B39	3	B64	2
A29	5	B41	2	B65	3
A30	5	B42	3	B67	2
A31	3	B44	3	B71	3
A32	5	B45	2	B72	3
A33	4	B46	2	B73	2
A36	3	B47	2	B75	2
A66	3	B48	2	B76	1
A68	6	B49	3	B78	1
A69	2	B50	2	B81	2
A74	3	B51	4	B8201	1
A80	2	B52	3		
		B53	3		
		B54	3		
		B55	3		
		B56	3		

HLA, human leukocyte antigen; SPI: solid-phase immunoassay.
Source: One Lambda Inc. (17).

were added, followed by incubation at 22°C for 90 min. The addition of stain and reading followed the same procedures as for the CDC assay.

CDC-DTT assay

CDC-DTT followed the same CDC protocol, and the only difference was the use of sera previously treated with DTT.

PRA detected by SPI

A commercial kit (LS1PRA[®], One Lambda, Inc.) was used according to the manufacturer's instructions to detect class I anti-HLA antibodies. The antigen panel used in this kit (17) is described in Table 2. Data were acquired using a LABScan[™] 100 unit (One Lambda, Inc.) and analyzed using the HLA-Visual[™] software.

As for the cut off point for definition of positivity in the SPI, reactions with median fluorescence intensity greater than or equal to 500 were considered positive.

Statistical Analysis

The McNemar, chi-square, and paired *t*-tests were used to compare and estimate statistical significance of results. The level of significance was set at 5% ($P < 0.05$).

TABLE 3. Comparison of Mean Differences Between CDC, CDC-AHG, and SPI

Differences	Mean	Standard deviation	<i>t</i> _{cal}	<i>P</i>
(CDC-AHG) – (CDC)	17.2	23.2	6.20	<0.0001
SPI – CDC	23.4	23.6	8.27	<0.0001
SPI – (CDC-AHG)	6.2	21.9	2.37	0.0208

CDC, complement-dependent cytotoxicity assay; CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; SPI, solid-phase immunoassay.

The efficiency of the methods used to detect anti-HLA antibodies was assessed using diagnostic tests and calculating sensitivity, specificity, positive predictive value, and negative predictive value, as well as false positive and false negative results.

Ethics

This study was approved by the Ethics in Research Committee of Universidade Estadual de Maringá (no. 212/2009), and all ethic procedures defined in Resolution 196/96 of the Brazilian National Health Council (18) were followed.

RESULTS

Analysis of PRA Detected by CDC, CDC-AHG, and SPI

The comparison of CDC, CDC-AHG, and SPI showed that, of the 70 serum samples, the highest mean positivity in detecting anti-HLA antibodies was found for the SPI test 37.5% (± 34.2), and percent PRA ranged from 0% (minimum value) to 98% (maximum value). In CDC and CDC-AHG, mean positivity was 14% (± 26.8) and 31.5% (± 37.5), respectively, and percent PRA ranged from 0% to 100% for both techniques.

The comparison of median values between the three methods also revealed that, in the SPI method, half of the serum samples had percentages equal to or lower than 28%, and the most frequent percent PRA was 0% (mode). The third quartile of the assays used showed that 75% of the samples analyzed using SPI had a percent PRA equal to or lower than 73%. The analysis of the third quartile of the other techniques showed a value of 63% for CDC-AHG and 21% for CDC without AHG (data not shown).

In addition to descriptive statistics, which analyzed continuous variables, mean differences were also compared between the three methods (paired *t*-test). Based on these data, this study found that, at a level of significance of 5%, there was a statistically significant difference when the mean difference was compared between the three methods (Table 3).

TABLE 4. Classification of Two of the Methods Under Study: CDC and SPI

SPI/CDC	Positive	Negative	Total
Positive	25 (a)	25 (b)	50
Negative	1 (c)	19 (d)	20
Total	26	44	70

CDC, complement-dependent cytotoxicity assay; SPI, solid-phase immunoassay.

TABLE 5. . Classification of Two of the Methods Under Study: CDC-AHG and SPI

SPI/CDC-AHG	Positive	Negative	Total
Positive	42 (a)	8 (b)	50
Negative	7 (c)	13 (d)	20
Total	49	21	70

CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; SPI, solid-phase immunoassay.

The McNemar test revealed that percent PRA detected by CDC and SPI was significantly different at a level of significance of 5% ($P < 0.0001$), which suggests that probability of positive results using the SPI is greater and confirmed that this method has a higher sensitivity than CDC without any additional treatments.

The diagnostic test used in this comparison confirmed the efficiency of SPI in the detection of anti-HLA antibodies. Sensitivity, specificity, positive predictive value, negative predictive value, false positive, and false negative values for the SPI, when CDC was used as the gold standard, were 96.2%, 43.2%, 50.0%, 95.0%, 50.0%, and 5.0%, respectively.

Only one of the serum samples of the 70 patients under analysis had a positive result by CDC and a negative result by SPI, which indicates that it was a false-negative case according to the diagnostic tests (Table 4, position c); however, previous analyses suggested that this patient had IgM isotype antibodies, which were, therefore, not detected by SPI.

The comparative analysis of CDC-AHG and SPI using the McNemar test showed that, at the level of significance of 5%, there were no significant differences between them ($P = 0.8026$). Sensitivity, specificity, positive predictive value, negative predictive value, false-positive and false-negative values for the SPI, when CDC-AHG was used as the gold standard, were 85.7%, 61.9%, 84.0%, 65.0%, 16.0%, and 35.0%, respectively. Later, serum treatment with DTT revealed the presence of IgM isotype in seven serum samples (Table 5, position c) negative by SPI and positive by CDC-AHG.

In addition, 18 serum samples had a higher percent PRA by CDC-AHG than by SPI (Fig. 1).

PRA Detected by CDC After Treatment With DTT

Previous treatment with DTT and subsequent testing with CDC-AHG revealed that 7 of the 70 samples under analysis were positive for IgM isotype. These results were confirmed using the SPI, which detects only the IgG isotype (Fig. 1).

DISCUSSION

Kidney transplants should be avoided in the presence of alloreactive cytotoxic antibodies due to the risk of hyperacute rejection (6). For this reason, crossmatching using the CDC microlymphocytotoxicity method remains widely used, even after the development of more sensitive methods (12).

The purpose of adding AHG to CDC is to eliminate low-affinity antibodies, amplify the cytotoxicity reaction promoted by low titer complement-fixing antibodies, and detect nonfixing complement antibodies, which increases the sensitivity of the method (4). As expected, the results of our analyses confirmed data found in the literature (19).

When PRA was analyzed using CDC-AHG after serum treatment with DTT (CDC-DTT), seven samples, previously positive, were negative, which suggests that the isotype detected before treatment was an IgM isotype. These results were confirmed using SPI, which detects only the IgG isotype (Fig. 1).

The DTT reduction agent enables the identification of the antibody isotype present in the patient's serum (IgM/IgG) (16). According to some authors, the presence of IgM isotype antibodies is associated with the production of autoantibodies, and there is no contraindication for transplantation in this case (8, 16). In contrast, the presence of IgG isotype antibodies against the specific donor cells is deleterious and may lead to hyperacute rejection (6, 7).

Some studies showed the importance of detecting IgM isotype antibodies and its association with graft survival. Yeğin et al. (20) reported the case of a patient who had positive CDC results that turned negative after the treatment with DTT. The patient received a living donor kidney transplant, and rejected 6 months after the transplant. The same authors reported another case, in which there were two positive CDC results that, after treatment with DTT, turned negative. In this case, the graft was preserved.

Khodadadi et al. (8) found convincing evidence that most IgM isotype antibodies do not play an important role in acute rejection. According to their results, 5.6% of the patients with IgM positive and IgG negative in crossmatching and PRA are expected to have a successful kidney transplant.

The action of IgM isotype antibodies in graft survival remains controversial, and it is fundamental to describe

Fig. 1. Percent PRA detected by CDC-AHG and SPI. The highlighted area (gray) corresponds to seven patients with positive results by CDC and negative by SPI. CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; PRA, panel reactive antibodies; SPI, solid-phase immunoassay.

this class of antibodies using methods such as CDC-DTT. Moreover, patients who have this type of antibody detected before transplantation should be more carefully followed up.

The comparative analysis of CDC and SPI revealed a significant difference ($P < 0.0001$), which indicates a higher chance of positive results when using SPI and its greater efficiency in detecting anti-HLA antibodies. Sensitivity (96.2%) was in agreement with the study conducted by Colombo et al. (21), who found a high percent PRA when SPI was compared with CDC.

The comparison of CDC-AHG and SPI did not reveal any significant differences ($P = 0.8026$), which indicates that these methods have similar diagnostic performances. Although not evaluated statistically, PRA values for 18 of the 70 samples were higher by CDC-AHG than SPI (Fig. 1). This finding may probably be associated with the difference in frequency of HLA specificities used in each method (Tables 1 and 2).

Sensitivity (85.7%), evaluated according to the comparison between CDC-AHG and SPI, was lower than the value found in the comparison between CDC and SPI.

In general, percent PRA was higher by SPI than CDC-AHG (Fig. 1). However, Gibney et al. (22) reached the conclusion that, although percent PRA by SPI is higher, each individual should be examined carefully, because data found in their study pointed to the little importance of a high percent PRA in the absence of DSA when associated with cases of acute rejection or excellent graft

survival. However, that same study suggested that, in the presence of DSA, SPI is a valuable test to identify possible clinical and immunological risks before transplantation.

Although current anti-HLA antibody detection methods are more sensitive and provide results in a shorter time, commercial kits do not identify isotypes (IgM/IgG) (15). In their review, Doxiadis, Roelen, and Claas (12) supported the use of the “old” CDC as the main method to define the level of sensitization and as an instrument to allocate kidneys for highly sensitized patients.

We understand that the CDC is a much more laborious method, which requires a panel of viable and available cells. However, this method has the advantage of reflecting a situation that more closely resembles the observed situation in vivo, because HLA, used as antibody targets, does not undergo manipulation.

We are also aware that problems involving cell viability, which include cell-based assays, have been solved with the introduction of solid-phase assays, since beads coated with HLA molecules are used as targets for antibody detection. However, based on our findings, we can state that, in order to detect percent PRA and distinguish between IgG and IgM antibodies, the CDC assay with the addition of AHG and DTT may still be an option in routine laboratory practice.

Undoubtedly, for the definition of anti-HLA antibody specificities, the SPI should be the method of choice. However, the purpose of this study was not to compare CDC assay sensitivity with a single-antigen assay, but rather

to the sensitivity of SPI-LS1PRA[®] (One Lambda, Inc.), which focuses more on sensitivity than on specificity in the detection of anti-HLA antibodies (23). We also performed this study to demonstrate that the CDC assay can continue to be used in routine laboratory procedures as a safe method to define the degree of sensitivity of patients on the waiting list for transplantation.

Of the limitations of our study, the small number of samples and the number of cells used to compose the T-cell panel for CDC were particularly relevant. Despite these limitations, data were enough for statistical analyses and purpose of this study.

CONCLUSION

Although CDC is an “old” method, the addition of AHG and the use of DTT led to the conclusion that its performance remains the criterion (gold) standard when compared with a more recent method (SPI-LS1PRA[®], One Lambda, Inc.) to assess percent class I PRA and to define sensitized patients.

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