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# Accuracy of the median channel shift in the flow cytometry for predicting complement dependent cytotoxicity crossmatching in kidney transplant candidates

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ABSTRACT

Crossmatching either by complement-dependent cytotoxicity (CDC) and/or by flow cytometry (FCXM) are routinely used for assessing anti-HLA donor antibodies before kidney transplantation. FCXM has demonstrated greater sensitivity and many transplant centers have opted for its use without the concomitant CDC assay. The objective of this study was to evaluate the accuracy of the median channel shift (MCS) in the FCXM in predicting the CDC assay results. A total of 1516 T cell FCXM and 1408 B cell FCXM were studied in deceased donors lymphocytes between January/2016 and March/2017. The high detection rate of CDC+ results by FCXM+ resulted in 87% (FCXM-T) and 90% (FCXM-B) sensitivity, and 98% negative predictive value, for both. The low specificity of FCXM B (43%) is atributed to cases of CDC-/FCXMB+. FCXM T and B were able to detect 53% and 76% of cases with donor specific antibodies of classes I and II with intensity of fluorescence ≥5001. The MCS differentiated CDC+ (Md, P25 and P75) results: MCS-T 390 (245–469) and MCS-B 282 (180–350). Through ROC curve analysis (AUC), the MCS showed satisfactory performance in detecting CDC+: MCS-T 0.909 (0.886–0.933) and MCS-B 0.775 (0.724–0.826). Considering the accuracy and sensitivity evaluation, the MCS-T 245 and MCS-B 282 cutoffs showed a better prediction of CDC+. This study showed that it is possible to calibrate MCS based on CDC+ with accuracy > 90%, however, that leads to a risk in terms of non-detection of low-titer anti-HLA antibodies.

## 1. Introduction

The complement-dependent cytotoxicity crossmatch (CDC) is still the gold standard technique for the pre-transplant evaluation of anti-donor antibodies in kidney transplantation its positivity contraindicates transplantation due to its strong association with hyperacute rejection [1–3].

The flow cytometry crossmatch (FCXM) is also widely used for the evaluation of the pre-transplant immunological risk with an admittedly higher analytical sensitivity [3–5]. As it is an automated technique, the definition of FCXM results is less prone to subjectivity than the assessment of cell death, as it occurs in CDC and presents good association with other tests such as solid phase assays [2,5,6]. The shorter technical execution time of the FCXM allows agility in the process of choosing the kidney recipient and consequently reduction of the cold ischemia time. In addition, not running CDC and FXCM crossmatches in the same evaluation reduces the number of tests and use of patient's sera. For these reasons, in the last decade many transplant centers, especially in North America, have chosen to replace the CDC relying only in the FCXM. However, this strategy bring out difficulties in interpreting the positive results, that are more frequent than in the CDC assays, but that do not necessarily contraindicate the transplantation

*Abbreviations:* CDC, complement-dependent cytotoxicity; FCXM, flow cytometric crossmatch; MCS, median channel shift; SA, single antigen; DSA, donor specific antibodies; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; DTT, dithiothreitol; AGH, anti-human globulin; AUC, area on the ROC curve; PCR-SSP, polymerase chain reaction - sequence specific primer; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value

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[2,5,7].

Studies evaluating CDC versus Single Antigen (SA) and FCXM versus Single Antigen in the context of virtual crossmatch are available in the literature, however studies evaluating the CDC versus FCXM are scarce. To our knowledge, only one study used the results of the CDC to define MCS cutoffs in the liver transplant scenario [8].

Therefore, we have evaluated the accuracy of the relationship between CDC and FCXM using the FCXM's median channel shift (MCS) measurement to predict CDC results.

## 2. Materials and methods

### 2.1. Study population

This is a retrospective study involving deceased donor kidney transplantation candidates at the Immunology Service of the Hospital de Clínicas de Porto Alegre. A total of 1705 crossmatches were tested in sera from 418 patients with different anti-HLA (human leukocyte antigen) antibodies levels against 237 donors lymphocytes, from January 2016 to March 2017. All sera, including current, peak and historical, were tested by FCXM, CDC and SA assays as described bellow.

### 2.2. Complement-dependent cytotoxicity crossmatch

CDC crossmatch was performed between donor T and B lymphocytes and recipient sera with and without dithiothreitol (DTT), ½ and ¼ dilutions, according to guidelines of the American Society for Histocompatibility and Immunogenetics [2]. CDC T had its sensitivity increased by the addition of Anti-Human Globulin (AGH). The reaction was stained with Fluoroquench® (One Lambda®) and visualized in an inverted phase microscope, and in the presence of ≥20% cell death the test was considered positive.

Out of the 1705 crossmatches CDC studied, 93 (5.4%) results were excluded from the analysis due to technical reading impossibility. For the purpose of this study, the following groups were analyzed: CDC T-/B-.

($n$ = 1312), CDC T+/B+ ($n$ = 204) and only CDC T-/B+ ($n$ = 96).

### 2.3. Flow cytometric crossmatch

FCXM was performed by using a modified Halifax [9] protocol. Lymphocytes were isolated from lymph nodes or spleens and treated with pronase (concentration 0.0235 U / mL). Then, 50 μL of pure serum from the recipient and 25 μL of donor lymphocyte suspension ($125–250 \times 10^3$ cells) were incubated for 15 min at room temperature. After 4 washes, the reaction mixture composed of anti-CD3/PerCP (BD Bioscience, clone SK7, catalog 340,663), anti-CD19/PE (Beckman Coulter, clone J3–119, ref. A07769) and Fragment F(ab')2 anti-IgG human conjugated to FITC produced in goat (Immuno Jackson Research, reference 109–096-098) was added and incubated for 10 min at room temperature. After the final wash, the samples were acquired at FACS Canto II (BD Biosciences) and for data analyses, the BD FACS Diva™ software (BD Biosciences) was used in the 1024 channel scale. By prior standardization of the laboratory, T and B cytometric crossmatch was considered positive when MCS ≥63 and ≥ 112 FITC anti-IgG fluorescence channels, respectively. These cutoffs were determined by evaluating an in house negative control serum from a male donor AB, Rh negative, PRA I and II = 0%, from the HCPA Hemotherapy Service, tested against 23 deceased donor cells from the routine.

In total, 1516 anti-T lymphocytes FCXM and 1408 anti-B lymphocytes FCXM were analyzed.

### 2.4. Anti-HLA antibody screening

Detection of anti-HLA class I and class II IgG antibodies was performed using the LabScreen Single Antigen kit (One Lambda®) according to the manufacturer's instructions. Samples were acquired in a Fluoroanalyzer Luminex®. Data analysis was performed using HLA Fusion 3.0 software (One Lambda®). Anti-HLA antibodies were considered present when the mean fluorescence intensity (MFI) was equal or greater than1000.

The presence or absence of anti-HLA class I or II donor specific antibodies (DSA) was defined by the comparison of the antibodies specificity identified through SA and the HLA genotypes class I (A, B, C) and class II (DRB1, DQB1) determined by polymerase chain reaction - sequence specific primer (PCR-SSP) in 1338 crossmatches; 367 crossmatches were not included in this analysis due to the lack of donor typing for the HLA-C and HLA-DQ loci. Additionally, when present, DSA were classified according to MFI level of ≤5000 and ≥ 5001.

### 2.5. Statistical analyzes

CDC were used as the gold-standard for the diagnostic evaluation of the FCXM. The values of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy as well as the relations among techniques were evaluated by using contingency tables and expressed as number of observations, percentages and 95% confidence intervals (CI). The comparison between the MCS medians of the groups CDC versus FCXM was performed by the Kruskal-Wallis test and the multiple comparisons by Dunn test with Bonferroni adjustment. To evaluate the MCS ability to predict CDC results, ROC curve analysis was builted and the cutoff indication with the highest balance between sensitivity and specificity was verified by the Youden index. The confidence intervals were calculated in the WinPepi software version 11.65 and all other analyzes were carried out in the Social Package Statistical Sciencies version 18.0. The statistical significance was established at a p level $< 0.05$.

### 2.6. Ethical aspects

The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre under the number 160357.

## 3. Results

### 3.1. Diagnostic characteristics of FCXM in relation to CDC

In order to compare the positivity frequency of anti-HLA antibody detection tests as well as to determine the diagnostic characteristics of FCXM in relation to CDC, the groups CDC negative and CDC positive

**Table 1**
Frequencies and diagnostic characteristics of FCXM in relation to CDC.

| | N total | CDC+/ FCXM+ | CDC+/ FCXM - | CDC -/ FCXM + | CDC -/ FCXM - | Sensitivity/ Specificity (%) | PPV/ NPV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|
| FCXM T | 1516 | 178 | 26 | 254 | 1058 | 87/81 | 41/98 | 81 |
| FCXM B | 1408 | 86 | 10 | 754 | 558 | 90/43 | 10/98 | 46 |

CDC were used as gold-standard.
Complement-dependent cytotoxicity; FCXM, flow cytometric crossmatch; PPV, positive predictive value; NPV, negative predictive value.

**Table 2**
Presence of Class I DSA in the CDC/FCXM groups

| | DSA I | | |
|---|---|---|---|
| | without DSA | DSA $I \leq 5000$ | DSA I ≥ 5001 |
| CDC-/FCXM- | | | |
| n | 758 | 56 | 11 |
| % | 91,3 | 31,3 | 5,9 |
| (95% CI) | (89–93) | (25–38) | (3 – 10) |
| CDC-/FCXM + | | | |
| n | 32 | 71 | 98 |
| % | 3,9 | 39,7 | 53,0 |
| (95% CI) | (3–5) | (32–47) | (45–60) |
| CDC + /FCXM- | | | |
| n | 17 | 3 | 0 |
| % | 2,0 | 1,7 | |
| (95% CI) | (1–3) | (1–5) | |
| CDC + /FCXM + | | | |
| n | 23 | 49 | 76 |
| % | 2,8 | 27,4 | 41,1 |
| (95% CI) | (2–4) | (21–34) | (34–48) |
| Total | 830 | 179 | 185 |

CDC, Complement-dependent cytotoxicity; FCXM, flow cytometric crossmatch; DSA, specific donor antibodies; CI, confidence interval.

(T + /B + and T-/B + ) were compared with the FCXM negative and FCXM positive groups (T and B), as shown in Table 1. The frequency of positive crossmatches by CDC and FCXM anti-T lymphocytes was 13% and 28% and anti-B lymphocytes was 7% and 60%, respectively, regardless DSA. The sensitivity of FCXM T and B was 87% and 90%, resulting in 98% NPV for both. FCXM T detected a higher number of positive results compared to CDC (17% versus 13%) and the same was observed in FCXM B (54% versus 7%), which resulted in low PPV. Among the 1312 CDC B negative crossmatches, 754 (57%) resulted positive by FCXM B accounting the low specificity observed (43%).

### 3.2. Relationship of CDC and FCXM with presence of DSA

In order to evaluate the high prevalence of CDC-/FCXM + cases, we analyzed the association between presence and absence of DSA and the combinations of CDC and FCXM results (Tables 2 and 3). The absence of DSA class I was associated to 91.3% of the negative crossmatches for both techniques; on the other hand, 53% of cases with DSA class $I \geq 5001$ were detected only by FCXM T.

Regarding the associations with DSA class II, it was observed that absence of DSA was equally prevalent between the CDC-/FCXM- and CDC-/FCXM + groups (398/825). However, once a DSA class II was present, CDC-/FCXM + was able to identify more cases of DSA than the CDC + /FCXM + groups (180/263 versus 31/263).

**Table 3**
Presence of Class II DSA in CDC/FCXM groups

| | DSA II | | |
|---|---|---|---|
| | without DSA | DSA II ≤5000 | DSA II ≥5001 |
| CDC-/FCXM- | | | |
| n | 398 | 43 | 7 |
| % | 48,2 | 31,6 | 5,5 |
| (95% CI) | (45–52) | (24–40) | (2 – 11) |
| CDC-/FCXM + | | | |
| n | 398 | 83 | 97 |
| % | 48,2 | 61,0 | 76,4 |
| (95% CI) | (45–52) | (52–69) | (68–83) |
| CDC + /FCXM- | | | |
| n | 6 | 1 | 1 |
| % | 0,7 | 0,7 | 0,8 |
| (95% CI) | (0–2) | (0–4) | (0–4) |
| CDC + /FCXM + | | | |
| n | 23 | 9 | 22 |
| % | 2,8 | 6,6 | 17,3 |
| (95% CI) | (2–4) | (3 – 12) | (11–25) |
| Total | 825 | 136 | 127 |

CDC, Complement-dependent cytotoxicity; FCXM, flow cytometric crossmatch; DSA, specific donor antibodies; CI, confidence interval.

### 3.3. Characterization of CDC/FCXM groups in relation to MCS

Because of the higher analytical sensitivity of FCXM, the median values, P25 and P75 of the MCS T and B that characterize the positive CDC were determined (Table 4). The CDC + /FCXM + and CDC-/FCXM + groups had statistically different MCS medians for both T and B tests (MCS T: 390 and 162; MCS B: 282 and 179). The MCS median of the CDC + /FCXM- and CDC-/FCXM- groups were not statistically different for both T and B tests.

### 3.4. Relationship between MCS and CDC values by ROC curve analysis

ROC curve analysis was performed in order to better evaluate the performance of MCS value to predict the CDC results.

As shown in the Fig. 1 the cutoff point with highest youden index was a MCS of 63 channels (AUC: 0.909 (0.886–0.933)) for the T cell FCXM and 238 (AUC: 0.775 (0.724–0.826)) for the B cell FCXM.

In addition, different cutoffs were tested based on the MCS distribution of the CDC + /FCXM + group (Table 4) and the respective diagnostic parameters are shown in Table 5. The cutoffs for MCS $T = 245$ and MCS B = 282 were the points that showed higher accuracy without considerably impairing sensibility (Table 5).

## 4. Discussion

Historically, the practice of kidney transplantation was based on the condition of a negative CDC crossmatch. Over time, it was observed that even the transplants performed in such condition could also evolve with unfavorable immunologic outcomes due to the presence of donor anti-HLA antibodies [1]. Therefore, higher sensitivity tests for detection of anti-HLA antibodies became necessary to decrease the incidence of antibody-mediated rejections. Nowadays, in a large part of transplant centers, solid organ allocation invariably involves solid phase assays, which represents a paradigm shift as to the importance of traditional CDC assay [10–12]. In that context, FCXM is a test that detects complement-fixing and non-complement-fixing antibodies and carries advantages such as increased sensitivity, automation, quicker results and lower cost. In contrast, the difficulties in the technical standardization and the high background due to antibody binding to B lymphocytes surface are limitations of the test [3]. Therefore, the objective of this study was to evaluate of the MCS approach to predict CDC results, aiming at the exclusive use of FCXM as a pre kidney transplant test.

The higher antibody detection capacity of FCXM compared to CDC was evident in this study by comparing the prevalence of positive results by FCXM and CDC. Regarding the diagnostic characteristics, the high detection rate of CDC + results by FCXM + resulted in good sensitivity values and, consequently, excellent NPV.

On the other hand, it was observed that 2% of the negative FCXM turned out to be CDC + . The positivity only detected by the CDC is documented in the literature and may be attributed to the susceptibility of lymphocytes from the deceased donor to rabbit complement due to time of donor lymphocyte harvest up to the timing of the crossmatching [13]. Alternatively it can be caused by auto-anti-HLA antibodies IgM or IgM-anti-non-HLA antibodies, which are considered of low risk [2,14,15]. Importantly, non-HLA antibodies directed at specific tissue auto-antigens expressed in endothelial and epithelial cells would not explain the CDC only positivity, since such targets are not expressed on the surface of lymphocytes [5]. In addition, kidney transplantation

**Table 4**
MCS value in CDC/FCXM groups

| | CDC + /FCXM + | CDC + /FCXM- | CDC-/FCXM + | CDC-/FCXM- | *p*-value |
|---|---|---|---|---|---|
| MCS T | | | | | |
| n | 178 | 26 | 254 | 1058 | < 0.001 |
| Md (P25-P75) | 390[a] (245–469) | 23[b] (0,25–38) | 162[a] (105–266) | 1[b] (−9–11) | |
| MCS B | | | | | |
| n | 86 | 10 | 754 | 558 | < 0.001 |
| Md (P25-P75) | 282[c] (180–350) | 66[d] (43–88) | 179[c] (141–238) | 61[d] (35–87) | |

CDC, complement-dependent cytotoxicity; FCXM, flow cytometric crossmatch; MCS, Median Channel Shift; Md, median; P, percentile.
[a] The comparison of the medians between the CDC + /FCXM + and CDC-/FCXM + groups (p = .02).
[b] comparison of medians between the CDC + /FCXM- and CDC-/FCXM- ($p$ = .06) groups.
[c] and comparison of the medians between the CDC + /FCXM + and CDC-/FCXM + groups ($p$ < .001).
[d] comparing the medians between the CDC + /FCXM- and CDC-/FCXM- ($p$ = 1.00) groups.

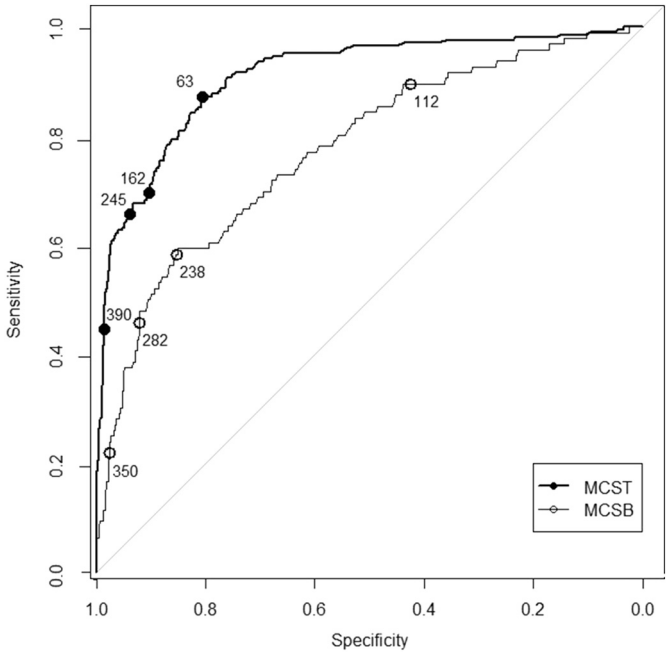


**Fig. 1.** ROC curve analysis between MCS and CDC. MCS T vs. CDC T ($n$ = 1516) present AUC = 0,909 and MCS B vs. CDC B ($n$ = 1408) present AUC = 0,775.

**Table 5**
MCS cutoffs tested by ROC curve

| | n | Sensitivity (%) | Specificity (%) | NPV (%) | PPV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|
| MCS T | 1516 | | | | | |
| 63 | | 87 | 81 | 98 | 41 | 81 |
| 162 | | 70 | 90 | 95 | 53 | 87 |
| 245 | | 66 | 94 | 95 | 63 | 90 |
| 390 | | 45 | 99 | 92 | 84 | 91 |
| MCS B | 1408 | | | | | |
| 112 | | 90 | 42 | 98 | 10 | 46 |
| 238 | | 58 | 85 | 96 | 22 | 83 |
| 282 | | 47 | 92 | 96 | 30 | 89 |
| 350 | | 22 | 98 | 95 | 40 | 92 |

MCS, Median Channel Shift; PPV, positive predictive value; NPV, negative predictive value.

performed in the presence of CDC AGH positive crossmatch with a negative FCXM and in the absence of DSA have been reported [14,16]. Graff et al. analyzed the data reported by the United Network for Organ Sharing (UNOS) between 1995 and 2009, evaluating 24-h, 1-year and 5-year grafts survival with living and deceased donors who presented CDC + /FCXM-. In comparison with a CDC-/FCXM- group no reduction in graft survival was observed suggesting the irrelevance of only CDC positive tests in this setting [14].

The occurrence of positive crossmatches exclusively by FCXM, resulted in low specificity and PPV. The exclusive positivity in FCXM B is widely documented in the literature, however, its clinical relevance is still controversial [7,17–21]. The high background of FCXM B can be due to the binding of immunoglobulins to IgG Fc receptors expressed on the surface of B lymphocytes [22], this partly accounts for the low predictive capacity of CDC B positive found in this study. In order to clarify the high prevalence of FCXM T and B positive and CDC negative, an analysis of these results was performed with an evaluation of presence of DSA. The presence of DSA I was more associated with FCXM *t*-tests than with CDC T + /B + , likewise the presence of DSA class II that was associated with cases of FCXM B compared to results of CDC B. In support, higher frequency of fluorescence levels of DSA classes I and II (≥ 5001) were present in FCXM T and B as compared with CDC T and B. Therefore, it seems plausible that FCXM is the safest cell test for pre-transplantation evaluation due to its sensitivity that is closer to the solid phase assays. On the other hand, we observed that the high rate of FCXM B positive in the absence of DSA class II impaired the specificity of FCXM B in relation to the CDC. Factors that may have contributed to this result include the absence of DP typing, the absence of epitope analysis and the limitations of SA [11]. This last, together with the analysis of DSA at a non-allelic level could explain 5.9% cases of DSA class $I \geq 5001$ which had negative crossmatches.

MCS analysis allowed the distinction of CDC + /FCXM + and CDC-/FCXM + groups with little overlap in terms of T and B channel deviations (21 and 58 channels). Interestingly, the median of MCS in the CDC + /FCXM- group was not significantly different from the MCS in the CDC-/FCXM- group. In addition, the low prevalence of this result, together with the absence of DSA classes I and II reinforces the notion that CDC positivity, in this scenario, is associated with IgM antibodies.

The ROC curve showed that the MCS presented a satisfactory performance for the prediction of CDC results, especially for FCXM T, which presented better prediction values than FCXM B. The MCS T and B points with the best balance between sensitivity and specificity were 63 and 238 channels, respectively, resulting in good accuracy values (81% and 83%). However, according to the characteristics of the CDC + /FCXM + group (Table 4) other MCS cutoffs were tested. It was possible to observe that the MCS accuracy increases according to the cutoff value tested, increasing FCXM specificity, but with a consequent sensitivity reduction, which can be explained by the choice of a gold standard of lower analytical sensitivity. Therefore, it is possible to predict positive CDC results in terms of MCS with accuracy > 90%, however, this posess a risk in terms of non-detection of low titer antibodies, as evidenced by the association between FCXM and DSA. Taking this fact into account, MCS T and B values of 245 and 282, respectively, presented satisfactory diagnostic characteristics for prediction of CDC + .

Some limitations apply to this study, the results could have been

strengthened by correlating the defined MCS values with post-transplant clinical data. However, only a fraction of the crossmatches resulted in transplants. Additionally, the analysis of DSA by epitopes could help elucidate the results.

In conclusion, the exclusive use of FCXM as a cell test for pre transplantation evaluation of anti-donor antibodies is feasible given the safety in terms of predicting CDC negative results and by assessing the risk of a preformed DSA. The NPV = 98% evidenced in this study demonstrates that the results of negative FCXM are truly negative when the permissible risk for transplantability is CDC-. Therefore, for this condition, FCXM's replacement of CDC becomes applicable and safe. Despite the low relationship with CDC B+, FCXM B it seems to be the most adequate test for DSA II evaluation. The semi-quantitative use of MCS $T \geq 245$ and MCS B $\geq 282$ for prediction of CDC positive results was demonstrated. Further studies detailing the relationship with SA are necessary to confirm the MCS values proposed in this study.

## Conflict of interest

None.

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