The Halifax flow Crossmatch Protocol Results According to the HLA Class and MFI of the DSA

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ABSTRACT

Introduction: The main strategy to reduce the immunogenicity of transplanted grafts is to seek maximum compatibility between alloantigens in the donor-recipient pair. It is understood that the virtual crossmatch (VXM) can be a good tool in the evaluation of the donor / recipient pair and that a broader application of these protocols will improve the pre-transplant immunological risk assessment. The aim of this study was to correlate the physical flow crossmatches results, looking at different mean fluorescence intensity (MFI) values of the donor specific antibodies (DSA), and the probability of a positive crossmatch. We also aimed to validate this tool using a well standardized flow crossmatch protocol. **Methods:** We performed a total of 15,217 FCXM between 2015 and 2019. All were tested by the Halifax Flow Cytometer Crossmatch (FCXM) protocol, with cells from deceased donors and serum from renal recipients. For this analysis we selected only samples that had one or two DSA per locus (N = 1,081), when the MFI was above 1,000, and they were divided according to the allelic group recognized by the antibody (anti-HLA-A, B, C, DR or DQ) looking at the probability of a positive crossmatch with different MFI values of the DSAs. Combinations among them were also analyzed in the same way (N=175). **Results:** In the presence of an exclusive DSA against the allelic groups A, B and DR, with an index MFI above 5,000, all the FCXM were positives. With exclusively antibodies against groups C or DQ, all cases with DSAs above 15,000 MFIs were positives. With two or more DSAs anti A and/or B, and/or DR, when their MFIs sums exceeded 5,000, all FCXM results against B cells were positive. The presence of anti-Class I DSAs (A, B and A+B), regardless of the MFI value, was responsible for 71% (N=424 of 601) of the T cell positivity and 77% (N=460 of 661) in the B cell crossmatches. The presence of only anti-Class II DSAs (DR and DQ) accounted for 55% (N=168 of 303) of FCXM positivity in B cells. The overall mean of the MFI of the DSAs was higher in the FCXM positive group when compared to negative crossmatch. The group with sum of DSAs A (N=217) showed that when the sum was 4,000 MFI or higher, there was a 24 times higher probability for a positive B cell crossmatch when compared with lowers MFIs. **Conclusions:** Our results show a strong association between the DSA MFI and the FCXM result. The data here presented confirm the results of our previous studies, justifying the VXM standard used by our center, proving to be a good tool to streamline the selection of transplant recipients and facilitate the sharing of organs from national donors.

Descriptors: Virtual Crossmatch; Flow Cytometer Crossmatch, Single Antigen Beads; Halifax Protocol.

Resultados da Prova Cruzada por Citometria de Fluxo pelo Protocolo Halifax de Acordo com o MFI do Anticorpo e o Alvo HLA

RESUMO

Introdução: A principal estratégia para reduzir a imunogenicidade dos enxertos transplantados é buscar a máxima compatibilidade entre os aloantígenos no par doador-receptor. Entende-se que a prova cruzada virtual (VXM) pode ser uma boa ferramenta na avaliação da dupla doador/receptor e que uma aplicação mais ampla desses protocolos melhorará a avaliação do risco imunológico

pré-transplante. O objetivo deste estudo foi correlacionar os resultados das provas cruzadas por citometria de fluxo (FCXM), observando os diferentes valores médios de intensidade de fluorescência (MFI) e o alvo dos anticorpos doador específicos (DSA), com a probabilidade de uma prova cruzada positiva. Usamos para isso o protocolo Halifax de prova cruzada por citometria de fluxo. **Métodos:** Foram realizados um total de 15.217 FCXM entre 2015 e 2019. Todos foram testados pelo protocolo Halifax Flow Cytometer Crossmatch (FCXM), com células de doadores falecidos e soro de receptores renais. Para esta análise, selecionamos apenas as amostras que apresentavam um ou dois DSAs por locus (N = 1.081) quando o MFI estava acima de 1.000. Esses foram divididos de acordo com o grupo alélico reconhecido pelo anticorpo (anti-HLA-A, B, C, DR ou DQ) observando a probabilidade de uma prova cruzada positiva com diferentes valores de MFI dos DSAs. As combinações entre eles também foram analisadas da mesma forma (N=175). **Resultados:** Na presença de um DSA exclusivo contra os grupos alélicos A, B e DR, com índice MFI acima de 5.000, todos os FCXM foram positivos. Com anticorpos exclusivamente contra os grupos C ou DQ, todos os casos com DSAs acima de 15.000 MFIs foram positivos. Com dois ou mais DSAs anti A e/ou B, e/ou DR, quando suas somas de MFIs excederam 5.000, todos os resultados de FCXM contra células B foram positivos. A presença de DSA anti-Classe I (A, B e A+B), independentemente do valor de MFI, foi responsável por 71% (N=424 de 601) da positividade de células T e 77% (N=460 de 661) nas células B. A presença apenas de DSAs anti Classe II (DR e DQ) foi responsável por 55% (N=168 de 303) da positividade FCXM em células B. A média geral do MFI dos DSAs foi maior no grupo FCXM positivo quando comparado ao crossmatch negativo. O grupo com somatória de DSAs A (N=217) mostrou que quando a soma era de 4.000 MFIs ou mais havia uma probabilidade 24 vezes maior de uma prova cruzada positiva de células B quando comparado com MFIs menores. **Conclusões:** Nossos resultados mostram uma forte associação entre o DSA MFI e o resultado do FCXM. Os dados aqui apresentados confirmam os resultados dos nossos estudos anteriores, justificando o padrão VXM utilizado pelo nosso centro, revelando-se uma boa ferramenta para agilizar a seleção de receptores de transplantes e facilitar a distribuição de órgãos de dadores nacionais.

Palavras-chave: Prova Cruzada; Citometria de Fluxo, Single Antigen Beads; Protocolo Halifax.

INTRODUCTION

In addition to a better HLA compatibility between the donor-recipient pair for the prophylaxis of acute and chronic rejections, it is essential to assess the presence of specific anti-HLA antibodies (DSA) against the donor's antigens.[1]

The introduction of the complement - dependent cytotoxicity (CDC) assay as a standard test for the detection of pre - transplant anti - HLA antibodies was the starting point that culminated in the subsequent improvement of immunogenic tests. The flow cytometry crossmatch using the Halifax protocol (FCXM), as well as the panel reactivity by single antigen beads (SAB) and the improvement of the HLA typing, are today the main tools resulting from this evolutionary journey. The work of Patel and Terasaki in 1969 was undoubtedly the incitement that launched the field of human histocompatibility clinical tests, paving the way for the development of virtual crossmatch (VXM).[1–3]

The FCXM assay using the Halifax/Halifaster protocol is significantly more sensitive than cytotoxic assays, allowing for better detection of low-level HLA antibodies, thus improving the assessment of pre-transplant immune risk.[4–6]

The VXM represents the latest evolution in the analysis of donor-specific anti-HLA antibodies (DSA). It combines the antibody profile (Ab) of the recipient compared to the donor's HLA antigens (Ag) to determine the presence of the DSA and predict the immunological risk for a donor-recipient pair. Using this tool, it is possible to foresee not only the possibility of a negative FCXM, but also to quantify the probability of an actual positive test. Its importance is to reduce the test time and, consequently, to reduce the cold ischemia time. In many cases the VXM can be the only pre-transplant crossmatch, reducing unnecessary testing and also reducing laboratory expenditures.[2,7]

The parameters for a positive VXM varies depending on the conditions determined by each laboratory, it means the threshold to distinguish between negative and positive and the risk tolerance of the transplant center. In our hospital, we have a well-established cutoff point for positive VXM. DSAs with MFI equal to or greater than 5,000 against HLA A, B and or DR antigens and equal to or greater than 15,000 for HLA DQ are considered positive VXM based on our physical flow crossmatch experience and therefore, since 2011, we have not performed kidney transplantation in recipients with one or more DSA above these values.[8] Our hospital offers a deceased donor kidney for all patients, as long as the FCXM is negative, regardless of the presence of DSA below the values stipulated for the VXM. This definition is of fundamental importance, since it increases the access of our patients to the transplant, depending on a negative FCMX and not on a total absence of DSAs. Kidney transplants performed under these parameters have the same safety as transplants performed in their absence.[8–10]

The aim of this study was to correlate the physical flow crossmatch results performed in the presence of DSAs, with different MFI values, and the probability of a positive and negative crossmatch due to these DSAs. For this, we used the Halifax Protocol, a well validated and standardized flow crossmatch protocol, as a tool to achieve this objective.

MATERIALS AND METHODS

We performed a total of 26,316 crossmatches between 2015 to 2019, including VXM and FCXM. Of these, 15,217 were tested by the Halifax Flow Crossmatch Protocol, with cells from deceased donors and serum from renal recipients. For this analysis we selected only samples that had one or two DSA per locus (N = 1,081) and they were divided according to the allelic group in anti-HLA-A, B, C, DR or DQ DSAs. Also, different combinations among them were also analysed (N=175). The flow chart is shown in Fig. 1. We included all antibodies with a mean fluorecence of 1,000 and above.

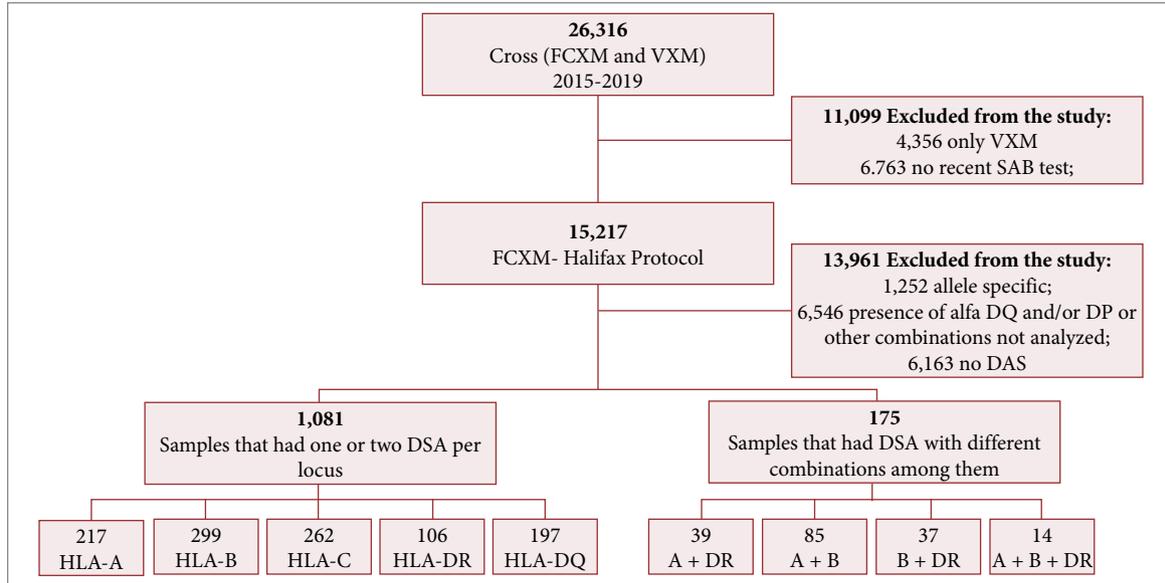Sera with allele specific antibodies were excluded and we used the mean MFI in the analysis.

Figure 1. Flow chart showing the cohort for this study.

Characterization of DSA

All antibodies were defined using the SAB technique using the Luminex platform (LABScreen SAB luminex assay OneLambda, USA) and analyzed using the HLA FUSION software (One Lambda, USA). Positive antibodies were considered when the MFI was above 1,000. All patient serum samples were heat treated (56°C) for 1 minute before the SAB test. We searched for DSA specific against HLA-A, B, C, DR and DQ. Panel Reactive Antibodies calculation (cPRA) was based on the HLA profile of a reference cohort of actual 2,000 renal donors from our region and results were expressed in percentages.

The DSAs were categorized by MFI range: 1.000 to 1.999, 2.000 to 4.999 and equal or above 5,000 for HLA A, B and DR groups. For the HLA C and HLA DQ groups the range above 15,000 was also added. The DSA MFI average of overall and each group were calculated.

HLA typing

HLA typing was performed using PCR Micro SSP DNA Typing Trays (Sequence Specific Primers-One Lambda, USA) or LABTypeTM SSO Typing Test (sequence specific oligonucleotide - One Lambda, USA). The tests were performed using the Luminex platform (LABScreen SAB luminex assay OneLambda, USA) and analyzed using the HLA LABTYPE software (One Lambda, USA).

The Flow Crossmatch Test

FCXM was performed using the Halifax protocol. All sera analyzed in the pre-transplant were also tested by SAB.[5,11]

The cells of the deceased donors were isolated from lymph node or spleen samples. Lymphocytes were separated by density gradient and treated with Pronase (0.5 mg/mL, Sigma, St Louis, USA). Receptor cells and serum were incubated and then labeled with an anti-CD3 (PerCP), anti-CD19 (PE), both from BD Biosciences, and anti-IgG (FITC) from Jackson Laboratories. The analysis was performed on FACSCanto II (BD, Franklin Lakes, NJ, USA). The positive reaction was determined by calculating the mean channel shift compared to negative control. The cut off is 75 and 115 channels shift for T and B cells respectively, calculated based on the average fluorescent channel obtained from 2,400 crossmatches performed with real waiting list patients with no DSAs against the cell used, plus 2.5 standard deviations.

The channel shift (CS) average was also calculated when the FCXM result was positive.

Statistical Analysis

All analyzes were performed using the Statistical Package for Social Science (SPSS, version 23) software. Statistical significance was assessed using linear regression, Chi-square or Fisher's Exact, Student's T or Wilcoxon-Mann-Whitney. Values for P≤0.05 were considered significant.

RESULTS

The presence of DSAs targeting just one locus was found in 1,081 sera. Of these, 217 (20.1%) had DSA against HLA A, 299 (27.6%) against HLA B, 262 (24.2%) against HLA C, 106 (9.8%) against HLA DR and 197 (18.2%) against HLA DQ (Fig. 2). Sera with two or more DSAs against different loci totaled 175 samples, with 85 (49%) anti-A and B, 39 (22%) anti-A and DR, 37 (21%) anti-B and DR and 14 (8%) anti- A and B and DR.
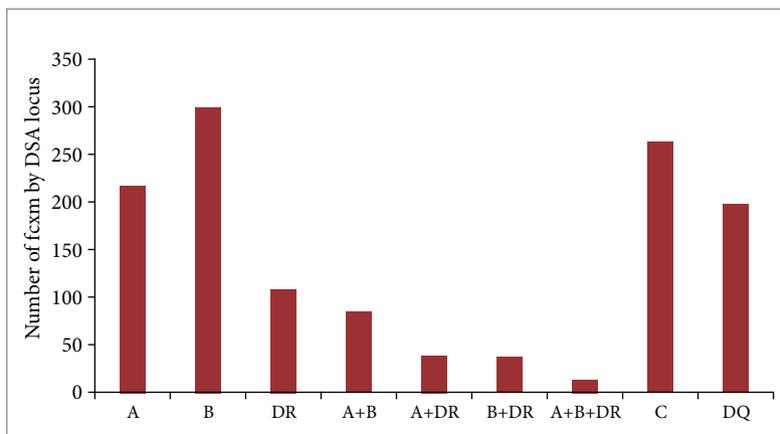

Figure 2. DSA prevalence by locus and prevalence of one or more than one DSA at different loci.

For all groups studied, an increase in CS was observed in the FCXM both in T and in B cells, according to the increase in the MFI. The exception was the anti HLA-C antibodies that did not showed a linear progression of CS with increased MFI.

In groups of anti A, B or DR, in the presence of an exclusive DSA above 5,000 MFI, all FCXM were positives. For groups anti-C and DQ, all cases with DSAs with MFI above 15,000 had a positive FCXM. In groups with two or more DSAs anti-Class I, when their sums exceeded MFI of 5,000, all FCXM results were positive (Fig. 3).
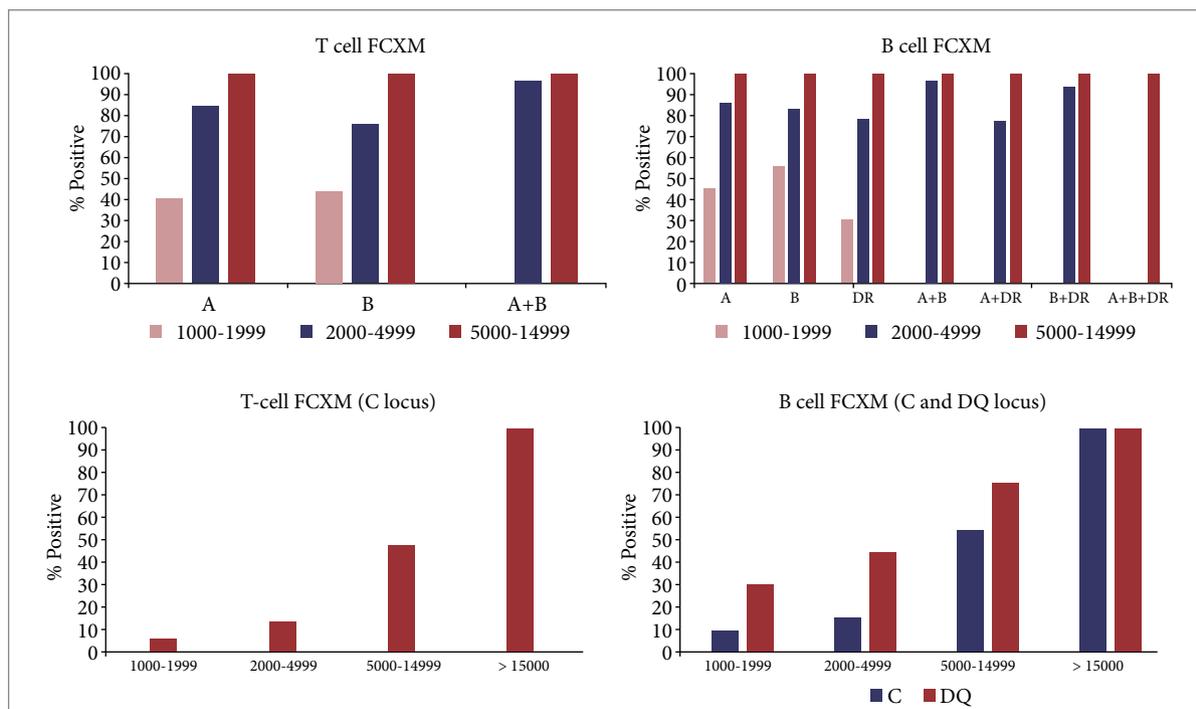

Figure 3. The relationship between CS and FCXM according to the increase in the MFI of the DSA.

The presence of anti-Class I DSAs (A, B and A+B), regardless of the MFI value, was responsible for 71% (N=424 of 601) of the T cell positivity and 77% (N=460 of 661) in the B cell crossmatches. The presence of only anti Class II DSAs (DR and DQ) accounted for 55% (N=168 of 303) of FCXM positivity in B cells.

The overall mean MFI DSAs were higher in the FCXM positive group when compared to the negative crossover tests, as expected.

The logistic regression analysis graph of the group with the sum of only DSAs is shown in Fig. 4. In the group with anti A, when the sum of the DSA reaches an MFI of 4,987, there is a 99% chance of a positive FCXM.
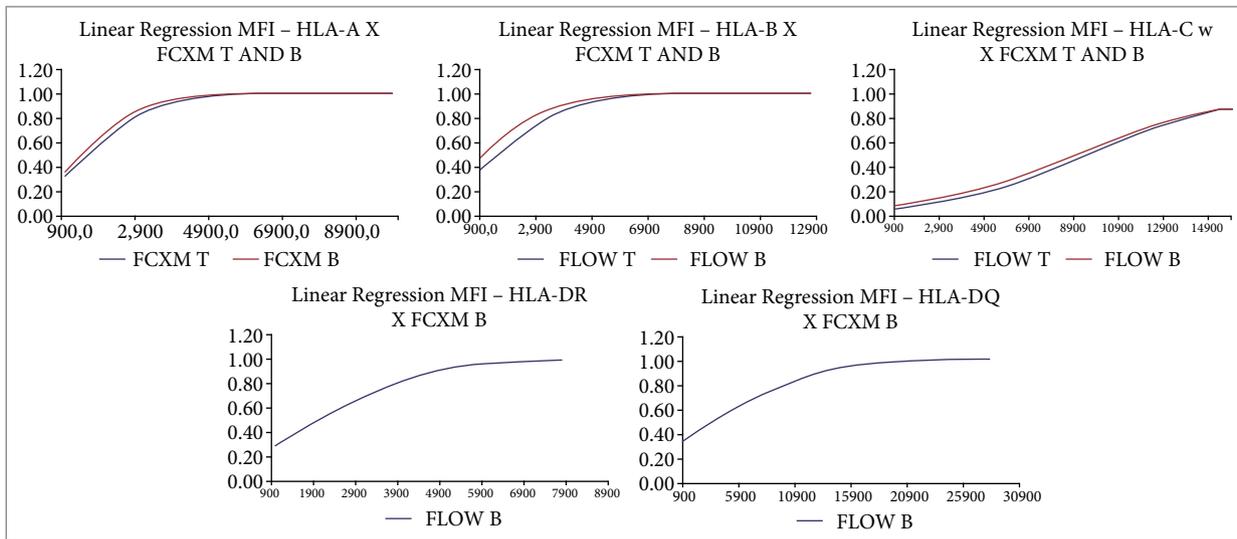
Figure 4. The logistic regression analysis graph.

Binary logistic regression was calculated to assess the association between the MFI of the DSAs and the result of the FCXM. Each group was analyzed within a given MFI range. In the groups with DSA HLA A, B and DR, the ranges were divided into: greater than 2,000, 3,000 and 4,000. In the HLA C and DQ groups, the MFI ranges above 5,000 and 10,000 were included. Table 1 shows the value of the odds ratio of each group. The group with sum of DSAs A (217) showed that in patients whose sum is greater than 4,000 they are 24 times more likely to have positive FCXM in B cells when compared to those with lower MFIs. The same occurs in all groups studied, with the values found being statistically significant (Table 1).

Table 1. The risk of a positive FCXM in the groups with different ranges of DSA HLA A, B and DR.

HLA	MFI	N	FCXM T			FCXM B		
			OR	CI 95%	P	OR	CI 95%	P
A	>4000	44	**13,9**	3,2 - 59,4	**0,000**	**24,0**	3,2 - 178,6	**0,002**
	>3000	74	**9,4**	3,8 - 23,1	**0,000**	**9,4**	3,6 - 24,7	**0,000**
	>2000	126	**9,3**	4,8 - 18,1	**0,000**	**9,3**	4,6 - 18,6	**0,000**
B	>4000	63	**6,5**	2,7- 15,7	**0,000**	**9,1**	2,7 - 30,0	**0,000**
	>3000	113	**7,0**	3,7 - 13,4	**0,000**	**7,7**	3,5 - 16,8	**0,000**
	>2000	179	**4,8**	2,9 - 8,1	**0,000**	**5,1**	2,9 - 8,9	**0,000**
	>10000	25	**24,6**	9,0 - 67,0	**0,000**	**18,2**	6,8 - 49,0	**0,000**
C	>5000	53	**10,4**	5,1 - 21,2	**0,000**	**10,0**	5,0 - 20,0	**0,000**
	>4000	68	**11,4**	5,5 - 23,5	**0,000**	**9,8**	5,0 - 19,1	**0,000**
	>3000	92	**8,8**	4,2 - 18,6	**0,000**	**7,6**	3,8 - 14,9	**0,000**
	>2000	133	**6,0**	2,7 - 13,6	**0,000**	**4,5**	2,2 - 9,0	**0,000**
DR	>4000	11	*	*	*	**5,4**	1,1 - 26,5	**0,036**
	>3000	22	*	*	*	**4,7**	1,6 - 14,1	**0,005**
	>2000	38	*	*	*	**9,9**	3,7 - 26,1	**0,000**
	>10000	50	*	*	*	**9,6**	3,6 - 25,6	**0,000**
DQ	>5000	97	*	*	*	**6,0**	3,2 - 11,4	**0,000**
	>4000	108	*	*	*	**4,2**	2,3 - 7,7	**0,000**
	>3000	127	*	*	*	**3,7**	2,0 - 6,9	**0,000**
	>2000	155	*	*	*	**4,4**	2,1 - 9,2	**0,000**

OR: odds ratio CI: confidence interval MFI: mean fluorescence intensity; FCXM: flow cytometry crossmatch T: T cells, B: B cells

DISCUSSION

The VXM is defined as "an assessment of immunological compatibility based on the patient's alloantibody profile compared to the donor's histocompatibility antigens.[2] More recently, due to improvements in HLA typing techniques and DSA identification, the VXM have considerable advantages over physical cross-matching. In many cases the VXM may be the only crossmatch needed or even possible in the pre-transplant setting.[2]

The availability of the VXM, sometimes suppressing the need for a prospective physical crossmatch, has been shown to decrease the cold ischemia time and therefore, the waiting list mortality for lung and heart transplant recipients. In addition, this tool plays an important role in increasing the likelihood of finding a suitable donor for hypersensitized recipients, resulting in successful transplants.[12–14]

The parameters for a positive VXM vary depending on the conditions determined by each laboratory, it means the threshold to distinguish between negative and positive and the risk tolerance of the transplant center. In our hospital, we have a well-established cutoff point for positive VXM. DSAs with MFI equal to or greater than 5,000 against HLA A, B and or DR antigens and equal to or greater than 15,000 for HLA DQ are considered positive VXM based on our physical flow crossmatch experience and therefore, since 2011, we have not performed kidney transplantation in recipients with one or more DSA above these values.[8] Our hospital offers a deceased donor kidney for all patients, as long as the FCXM is negative, regardless of the presence of DSA below the values stipulated for the VXM. This definition is of fundamental importance, since it increases the access of our patients to the transplant, depending on a negative FCMX and not on a total absence of DSAs. Kidney transplants performed under these parameters have the same safety as transplants performed in their absence.[8–10]

In cases of anti-HLA-C the results reliably predicted FCXM only with MFI values starting at 10,000, being 100% (Fig. 5).[8,15] The results here presented show a very good association between the DSA MFI value and the FCXM result, in agreement with the practice already adopted in our center.
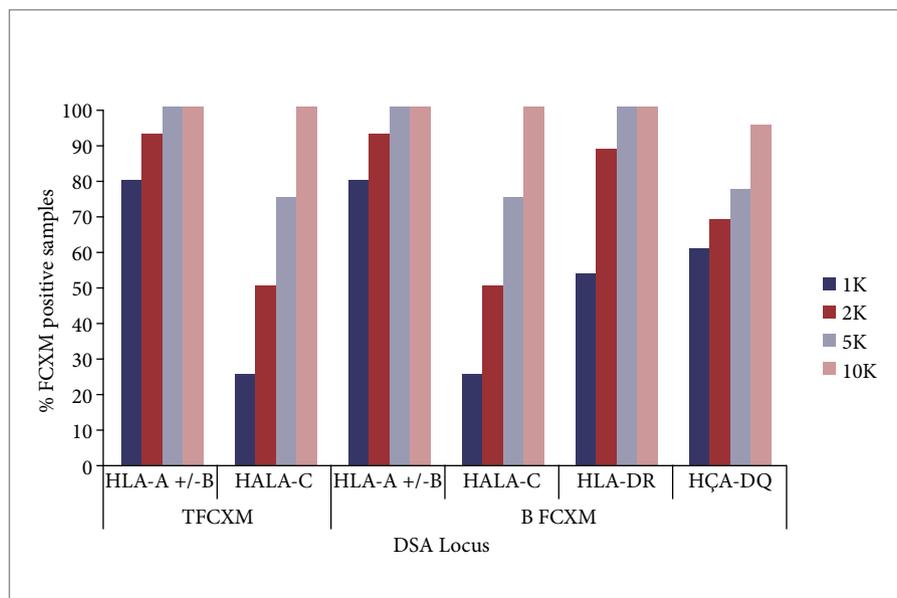

Figure 5. Correlation of virtual crossmatch and flow cytometry crossmatch in our previous study.

VXM: Virtual crossmatch; FCXM: Flow cytometry crossmatch

Although a lower expression level of HLA-C antigens, compared to HLA A and HLA B is well documented, we could speculate that HLA-C antigens might be less immunogenic than HLA-A and B, but they should always be considered, especially when detected pre transplant.[16] The occurrence of some FCXM reactions that did not respond in an MFI dependent way may be a reflection of the epitopic load from the foreing allele causing the immune reaction in relation to the host.[17,18] A more detailed study on the expression of each allele, as well as their epitopic load in relation to the host, must be carried out in order to observe its impact on the relation to FCXM. HLA expression is genetically regulated and can lead to determined variations in expression levels that may have clinical relevance.[16–19]

The results presented also show that in the presence of single-locus DSA A, B, C, DR and DQ with a value equal to or greater than 4.144, 4.502, 14.901, 4.563 and 14.587 MFI, respectively, have 100% specificity in FCXM B (ROC curve results, data presented in the Supplementary Table). Few studies are available providing data on the possible impact of different levels of HLA expression on the FCXM.[16–19] Our data shows that it is safe to predict a positive FCXM considering the above MFI values.

In our State in Southern Brazil (RS), 43% of transplants with deceased donors are from national donors offers, often associated with a long cold ischemia time and, consequently, we see a significant number of delayed graft function (DGF). Both, DSA and DGF are known risk factors for worse graft survival.[20] In this context, the VXM may be a good tool to streamline the

pre-transplant recipient selection and facilitate organ sharing over longer distances. It is noteworthy that VXM can be a barrier to transplantation depending on the cutoff point adopted by each center, especially when low MFI cutoff points are used to assign unacceptable antigens. In our center, we performed 162 kidney transplants with a deceased donor between 2020 and 2021. Of these, 43 (26.54%) came from national offers using the help of our VXM, that is, we do not accept donors against DSAs that will give a 100% positive physical crossmatch, and we do proceed with the transplant when we have a recipient with no detectable DSA, performing the physical crossmatch retrospectively. This procedure resulted in a very significant saving of time, resulting in shorter cold ischemia times on the virtual negative and a better destination for kidneys that could not be used by us due to a virtual positive crossmatch.[21]

Due to the complex nature of their interpretation, the boundaries of MFI are for the most part site-specific. As such, the variability of these thresholds can be attributed to testing protocols, laboratory analyst experience, and local clinical practice. It is of utmost importance to understand that the antibody binding to the bead or the secondary antibody binding to the anti-HLA antibody can be affected by different factors. For instance, it is known the role of Complement contributing to an inhibitory effect, colloquially called the "prozone effect". By treating the serum samples with ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) or heating before performing the assay (paper submitted), most of the inhibitory effect can be eliminated or substantially reduced.[22,23] Then, a careful and judicious correlation between the detection of the DSA becomes extremely important, both from the technical point of view of the SAB test and from the clinical point of view, in choosing the cutoff to determine an acceptable DSA.

On the other end of the spectrum, that is, what is the probability of a negative flow crossmatch in the presence of DSAs, we found that even in patients with a cPRA of 99 to 99.9% there is a probability of a negative physical crossmatch in the presence of a DSA anti HLA-A, B, C or DR, and that is the reason we do perform a physical crossmatch when the MFI of the DSA is between 1,000 and 4,999. Previous results from an ongoing study at our center showed no difference in patient and graft survival up to four years of follow up, as well as in renal function, in patients transplanted with or without DSA, as long as they were negative for FCXM.[9] This underscores our center's high sensitivity in predicting immunological match between donor and recipient, increasing access to the transplant for hypersensitized patients. Furthermore, the data here presented shows that DSAs anti-HLA A or B with an MFI up to 2,900 have a 20% chance of a negative physical flow crossmatch. Anti-HLA DR DSAs with this same MFI value are on average 35% negative, anti-HLA DQ 55% and anti-HLA C 85% negatives at this MFI value.

Furthermore, it also highlights the importance of correct standardization for analysis of the VXM and its correlation with FCXM, not only to detect a high risk DSA, but also to detect a weak and probably low-risk DSA. We believe that not all DSAs are the same and not all have the same immunopathogenicity. We consider that the presence of DSA against cryptic epitopes may explain some of our results, emphasizing the importance of epitopic analysis, which should be included on the VXM setup.[2,9]

Generally speaking, a negative VXM is associated with a negative FCXM and, inversely, high-titer DSAs is associated with a positive FCXM. However, it is important to be aware of uncommon cases, where there may be divergence not only in the linearity of the MFI x ratio and FCXM positivity, but also with false positive or negative results. In a false positive VXM, where we have the presence of DSA combined with a negative FCXM, one should identify patterns of denatured beads or cryptic antigens and implement epitope analysis to confirm the presence or absence of a true anti-HLA antibody. On the other hand, the reasons for false positive FCXM (in the absence of DSA) are not so easily identifiable. Tissue expression of HLA antigens can be variable and impossible to predict. Issues such as HIV infection, autoimmune diseases and immunosuppressive regiment can also lead to false-positive reactions and, alternatively, there is the rare possibility that a patient has an anti-HLA antibody against an allele not represented in current panel tests.[2] At our center, we have a very low false positive FCXM (2.6%).

Ideally, all DSA should be avoided, but this is often impractical in the context of a given transplant. Instead, the clinician must estimate the risk of graft rejection in each situation, against the risk of not being transplanted.[24,25]

Although some studies differ in their results, currently the strength of the DSA expressed in MFI at the time of transplantation is considered a risk factor for the occurrence of early AMR and allograft failure.[26,27]

Our criteria for VXM are illustrated in Fig. 6. We only exercise the virtual crossmatch in our transplants when we do have a donor typing that includes HLA-A, B, C, DR and DQ. This has become much more frequent in the last five years. As HLA-DP is not typed as routine in any solid organ donor in Brazil, when our recipient has anti HLA-DP we cannot do a virtual. With patients with a 0% cPRA the negative virtual crossmatch is assumed. The same when the most recent cPRA analysis (<120 days) with no immunizing event in this period, shows absence of any known DSA.
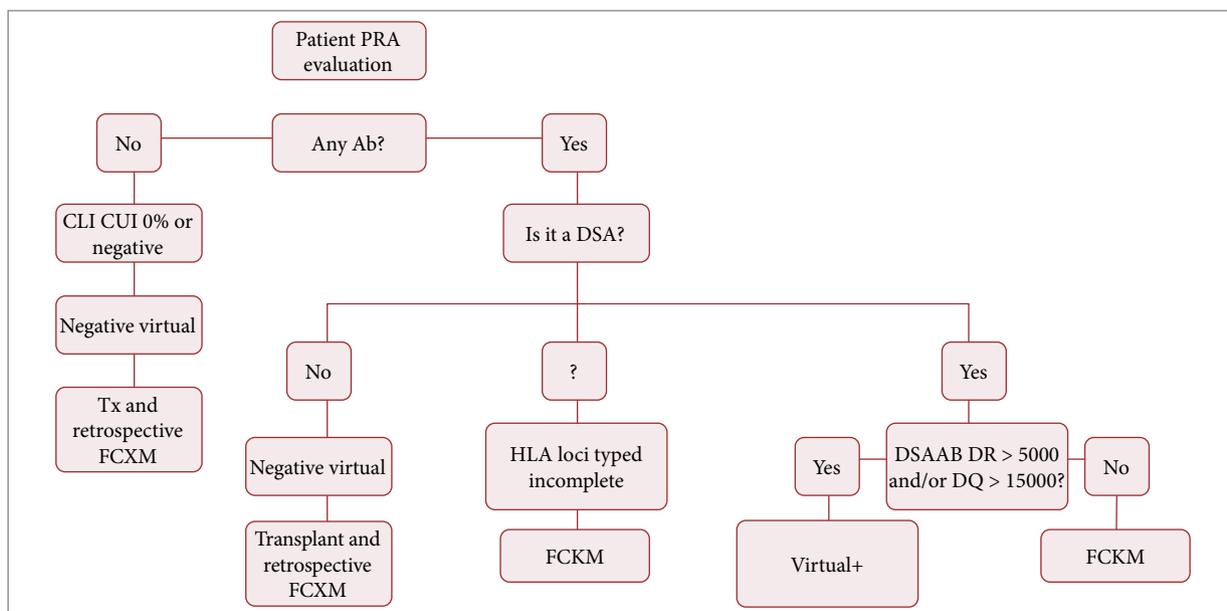
Figure 6. Santa Casa´s VXM strategy for risk stratification.

However, the physical crossmatch is performed even in the presence of high levels of DSAs in patients who have some type of medical urgency for kidney transplant. These are cases in which the medical team assesses the immunological versus clinical risk assuming the risk of transplanting even with a weakly positive FCXM. Those are situations in which the risk for the patient not to transplant is higher than the risk of rejection.

Considering the need to reduce pre-transplant testing time, we developed a computerized system for processing the relevant immunological data relative to the waiting list and a given deceased donor. The donor's HLA data is compared with the patient's anti-HLA antibody profile, aiming to assess whether they have any pre-formed DSAs and characterizing them according to our VXM algorithm (Fig. 5). Using this tool, we obtained a reduction of time from an average of 40 minutes (when done manually) to <5 minutes with the software (-82%), besides eliminating possible manual errors.[28]

Our center offers the opportunity for all patients on the list to receive a transplant regardless of the presence of DSAs, as long as the FCXM is negative or even positive under evaluation of the risk/benefit balance. The definition of the VXM is of fundamental importance, as it expedites the whole immunological evaluation and offers a tool for a better risk evaluation.[9,20]

CONCLUSIONS

Our results show a strong association between the DSA MFI and the FCXM result. The data here presented confirm the results of our previous studies, justifying the VXM standard used by our center, proving to be a good tool to streamline the selection of transplant recipients and facilitate the sharing of organs from national donors.

AUTHORS' CONTRIBUTION

Substantive scientific and intellectual contributions to the study: Schiavo, T.; Montagner, J.; Keitel, E.; Greenshields, A.L.; Liwski, R.S.; Neumann, J.; **Conception and design**: Schiavo, T.; Montagner, J.; Keitel, E.; Greenshields, A.L.; Liwski, R.S.; Neumann, J.; **Data analysis and interpretation:** Schiavo, T.; Liwski, R.S.; Neumann, J.; **Article writing:** Schiavo, T.; Greenshields, A.L.; Neumann, J.; **Critical revision:** Liwski, R.S.; Neumann, J.; **Final approval:** Neumann, J.

DATA AVAILABILITY STATEMENT

All data were generated in this study and are available under request.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest that could interfere with the results

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SUPPLEMENTARY TABLE

Table shows that the increase in the MFI of the DSA follows the increase in specificity in all groups. Specificity ranges were set at 75 and 100% and in groups with more than one DSA, 95 and 100%.

	MFI	FCXM T		Area	I.C. 95%
		Sensibility	Specificity		
A (N=217)	1878	0.233	0.750	0.800	0.740 - 0.860
	4827	0.890	1.000		
B (N= 299)	2321	0.335	0.750	0.741	0.685 - 0.798
	4742	0.838	1.000		
C (N= 262)	3162	0.0239	0.750	0.810	0.728 - 0.892
	14901	0.913	1.000		
DR (N= 106)	*	*	*	*	*
	*	*	*	*	*
DQ (N= 197)	*	*	*	*	*
	*	*	*	*	*

	MFI	FCXM B		Area	I.C. 95%
		Sensibility	Specificity		
A (N=217)	1803	0.240	0.750	0.804	0.744 - 0.863
	4144	0.740	1.000		
B (N= 299)	2302	0.369	0.750	0.744	0.685 - 0.804
	4502	0.815	1.000		
C (N= 262)	3036	0.278	0.750	0.782	0.703 - 0.862
	14901	0.926	1.000		
DR (N= 106)	1746	0.288	0.750	0.743	0.648 - 0.839
	4563	0.865	1.000		
DQ (N= 197)	4397	0.328	0.750	0.759	0.693 - 0.825
	14587	0.828	1.000		