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Lytic DSAs as the qualification criterion for virtual crossmatch test. Benefits and challenges

ABSTRACT

Pre-formed anti-HLA antibodies against potential donor antigens (donor-specific antibodies, DSA) are a prevalent risk factor significantly reducing the patient's chances of receiving a transplant. Pre-transplantation immunization assessments consist mainly of the high-sensitivity anti-HLA fluorescence flow cytometry assays (Luminex). The assays facilitate determination of the specificity of anti-HLA antibodies within the entire range of the IgG class, including the subclasses of non-lytic antibodies, such as IgG2 and IgG4, which are significantly less harmful to the transplant as compared to lytic antibodies. When the results of anti-HLA IgG assays are taken into account as the only qualification criterion for virtual crossmatching, without their lytic potential being determined, the recipient's chances of transplantation are significantly reduced. Given the increasing number of immunized patients, a modification of the virtual crossmatching protocol is proposed so that only recipients with anti-HLA antibodies identified as complement-binding [C1q(+)] DSAs are excluded from the further qualification process. The presence of C1q(−) DSAs would be an indication of an increased risk of humoral rejection rather than a contraindication for transplantation. The results of transplantations followed by strict monitoring of antibody levels in these patients are promising, albeit burdened by an increased risk of humoral rejection. This article presents benefits and challenges related to the introduction of the new algorithm focusing particularly on the interpretation of the C1q status of donor-specific antibodies.

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Key words: kidney transplantation, anti-HLA antibodies, complement binding anti-HLA, virtual cross-match, donor specific antibodies, transplant immunology

INTRODUCTION

Virtual crossmatch (vXM) based on the mean fluorescent intensity (MFI) has been in use in the Polish kidney allocation system since 2016. In its classic form, vXM absolutely prohibits a transplant being received from a deceased donor when the recipient presents with donor-specific antibodies (DSAs) against the human leukocyte antigen (HLA) within the HLA-A, -B, and/or -DR loci at MFI values of > 5000 as determined by the Luminex single antigen assays within the last six months [1]. According to the British Society for Histocompatibility & Immunogenetics and British Transplantation Society (BSHI/BTS) guidelines, DSA levels above this value are considered to be closely linked to the risk of humoral rejection [2].

Of the 2295 patients awaiting a kidney transplant in Poland, 619 recipients present with anti-HLA-A, -B, and/or -DR antibodies above the level of 5000 MFI. Approximately one-half of the subgroup of immunized subjects (333 individuals) simultaneously present with no detectable levels of lytic antibodies as determined by the panel reactive antibodies test with complement-dependent cytotoxicity (PRA-CDC = 0%) [3]. The extended donor HLA typing (-A, -B, -C, -DR, -DQ, -DP) introduced in 2002 as per the Poltransplant recommendations has facilitated obtaining full information regarding the risk associated with anti-HLA antibodies of class IgG detected in the recipient. The anti-HLA-Cw, -DQ, and -DP DSAs are currently not being taken into account for vXM purposes; however, such an

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extension would prevent another 480 subjects from getting a chance for being matched for transplantation, which means that they would have to remain on dialysis therapy.

The anti-HLA IgG fractions as determined using the solid-phase assay (L-SA) were shown to have no significant negative effect on transplantation, even from the 10-year follow-up perspective. When the above method is used as the qualification criterion for virtual crossmatching, the chances of receiving a transplant are greatly reduced for immunized patients. In view of the evident need for wider access to transplants, a novel algorithm [4] has been proposed; very simply put, the new algorithm replaces the vXM based on the DSA cut-off value of > 5000 MFI with the criterion based on lytic anti-HLA DSAs which bind the complement component C1q as measured using the Luminex assay (L-C1q). Only the presence of L-C1q(+) DSAs within the serum of the potential recipient would exclude him/her from further matching against the deceased donor, i.e. from biological crossmatching using a serological method (complement-dependent cytotoxicity crossmatch, CM-CDC) [4].

A populational screening for L-C1q antibodies had been performed in a group of 268 patients awaiting a kidney transplant at the Gdańsk kidney transplantation center [5]. MFI values were analyzed for all L-SA-detected specificities in undiluted sera with prozone effect reduction. About 40% of anti-class I and 30% of anti-class II antibodies were found to be lytic. The positive results of L-C1q assays were closely correlated with the MFI values as measured in L-SA assays ($R = 0.716$, $P = 0.001$ for Class I; $R = 0.791$, $P = 0.001$ for Class II). Regardless of the HLA class, most (97%) antibodies present at MFI levels of > 15000 were positive in L-C1q assays. With regard to antibodies above the current vXM cut-off level of > 5000 MFI, lytic antibodies accounted for 62% and 55% of anti-class I and anti-class II antibodies, respectively. Thus, about one-half of anti-HLA specificities detected in L-SA assays might not lead to the patient's exclusion from continuing with CM-CDC, which would deprive him/her of the chance to receive an organ transplant.

Would it be sufficient to avoid high L-SA results, such as those of > 15000 MFI while not assessing the L-C1q status?

The above proposal appears not to be a safe solution. As many as 15.5% and 9% of L-C1q(+) anti-HLA antibodies were detected below the level of 15000 MFI in class I and II, respectively. Most of the positive results of L-C1q assays corresponded to high L-SA levels of > 12000 MFI. A surprising finding consisted in the high percentage (amounting to approximately one-half — 54.3%) of lytic antibodies within the anti-HLA-DQ type. It was 20% higher than the overall percentage of lytic antibodies within class II (30%). Anti-DQ specificities detected above the LSA level of 13700 were L-C1q-positive. This relationship is observed in practice. Anti-HLA-DQ antibodies are very prevalent in patients awaiting re-transplantation; they may also be the reason for selective humoral rejection. The opposite is true for anti-HLA-DP antibodies; according to our results, only 8% of these antibodies are lytic. Consequently, they are not considered as per the Eurotransplant protocol.

The strengths and weaknesses of L-SA and L-C1q assays are presented below with an emphasis on their technical aspects facilitating their critical and objective assessment in the context of the results of relevant studies and implementation of the new algorithm to account for the presence of L-C1q(+) DSAs as the vXM criterion.

DIAGNOSTIC CHALLENGES CONCERNING ASSESSMENT OF HUMORAL RESPONSE BEFORE TRANSPLANTATION

In contrast to cell-mediated rejection, the risk of antibody-mediated rejection can be minimized by prospective anti-HLA antibody screening. In addition to the flow cytometry crossmatch (FCXM) as a well-established prospective biological crossmatch test, the pre-transplantation risk assessment is based on systematic fluorescence flow cytometry assays (Luminex). The assays are carried out in steps and can be evaluated in a multidirectional way. The first step is to determine the presence of anti-HLA antibodies (Luminex screen) along with determination of their class (I and/or II) while the second step consists of assessment of the specificity of these antibodies with semiquantitative determination in MFI units.

In many transplantation centers, including transplantation centers in Poland, the MFI value is used as quantitative parametr for the virtual crossmatch evaluation. In the absence of a better tool, the MFI level is treated as a quantitative measure. However, MFI is not part of the SI (International System of

▸▸ According to the BSHI/BTS guidelines, DSA levels above MFI = 5000 are considered to be closely linked to the risk of humoral rejection. ◂◂

▸▸ The anti-HLA IgG fraction as determined using the solid-phase test (L-SA) has no significant negative effect on transplantation, even from the 10-year follow-up perspective. When the above is used as the basis for virtual crossmatching, the chances for receiving a transplant are greatly reduced for immunized patients. ◂◂

Units) and is an indicative semi-quantitative measure. One may frequently encounter the statement that high MFI values are associated with large amounts of DSAs, whereas low MFI values are associated with no antibody-related risks. However, part of the results may be fraught with error.

False-negative results may be associated with the presence of inhibitors such as components of the endogenous complement systems or competently binding IgMs. The problem may affect up to 70% of patients with high levels of immunization with individual anti-HLA specificities [6]. A solution consists in appropriate preliminary analysis of the serum sample: the addition of ethylenediaminetetraacetic acid (EDTA) prevents the interference from the components of the endogenous complement by chelating the calcium ions within the serum. Dithiothreitol (DTT) reduces disulfide bonds present within interfering antibodies of the IgM class; thermal pretreatment eliminates the influence of the aforementioned interferents while increasing non-specific bonding and structurally denaturing the serum proteins. Measures to be taken to minimize the prozone effect are part of good laboratory practice, with most laboratories taking appropriate steps to prevent interference [7, 8]. However, problems related to the limited linearity of the Luminex method and the Hook effect, consisting of the antigen-antibody complex being formed only in conditions of relative equilibrium, remain unsolved. Another reason for underestimation of MFI values is the phenomenon of shared epitopes, i.e. the situation when the recipient responds to an epitope present on numerous antigens. Under the conditions of the L-SA assay, where the recipient's serum is tested against approximately 100 antigens in total, the antibodies uniformly attach to all antigens that bear the particular epitope. In such cases, the measured DSA-specific MFI value may be lower due to dispersion. *In vivo*, following the transplantation procedure, the recipient's antibodies have at their disposal only the epitope available within the donor HLA genotype which thus becomes the target of the immune response. Frequency of the specificity of in vitro-detected antibodies as provides a good example of antibody behawior. rovides a good example of antibody behavior. Thus, the antigen HLA-A80, while being rare within the population (0.09%) is detected in L-SA assays at a frequency of up to 27%, probably due to sharing some epitopes with the populationally prevalent HLA-A2 antigen [9]. A solution may be provided by performing serum dilution series to determine the antibody titers. Although this may increase the credibility of the MFI data, it is a rather expensive and hence infrequently practiced method. As L-SA assays are performed in sequential dilutions of the test serum, one may frequently observe antibodies with low MFI values as measured in native serum samples reaching their maximum intensities at lower concentrations. In highly immunized patients, some class anti-HLA antibodies within the IgG class reach their maximum intensities (MFI_{max}) in dilutions as low as 1:1024 [10].

Problems with the interpretation of the results, particularly in cases with the known cause of immunization, may arise due to false positives. These are related to a technological problem during the manufacture of solid phase tests. Partial denaturation of recombinant HLA antigens may occur as they are being coated onto the microspheres, resulting in generation of epitopes that are normally absent *in vivo*. The prevalence of antibodies against denatured HLA epitopes (anti-dHLA) is not low: 39% of 323 immunized patients presented with individual antibodies against denatured epitopes absent in the native antigen *in vivo*. False positive hits can be identified among detected antibodies by preincubating HLA microspheres in acidic conditions and repeating the L-SA assay. This method, however, has not been validated for broad clinical practice [11]. Another characteristic feature of the L-SA assay consists in a high coefficient of variation, with changes in MFI values considered to be significant only for values differing by about 25% [12].

CLINICAL RELEVANCE OF L-C1Q AND CHARACTERISTICS OF THE ASSAY

In vivo, the most aggressive anti-HLA damage mechanism involves activation of the classical complement pathway, the first stage of which consists in the C1q molecule being bound to the newly formed antigen-antibody complex (the IgG1, IgG3 subclasses). For the C1q molecule to bind to the newly formed complex, the presence of a minimum of 6 IgG antibody molecules within close proximity of the molecule is required to avoid accidental damage to a healthy cell. In other words, the level of antibody saturation must be high for a par-

ticular cell (or microsphere saturation in the *in vitro* setting) to result in C1q binding. Once bound, the C1q triggers the complement cascade sequence. The C4d and C3b proteins involved in the subsequent stages of the complement system activation are also used in *in vitro* diagnostics [13]. The first study conducted by Loupy et al. in a group of 1016 post-transplant patients showed that the emergence of complement-binding DSAs in the patient's serum was associated with poor prognosis [14]. Some of the authors draw conclusions on the usefulness of the clinical L-C1q assay with respect to determining the risk to transplant function, indicating that the presence of C1q(+) DSAs is associated with antibody-mediated rejection, glomerulopathy, and increased incidence of transplant loss. Patients who had DSAs before transplantation were subjected to desensitization (e.g. IVIG, plasmapheresis); even if the antibodies were completely removed, those patients remained at a higher risk of transplant rejection and loss as compared to non-immunized recipients [15–20]. Other authors remain skeptical as they find the additional assay failing to bring about clear benefits while pointing out that the same effects can be achieved by avoiding very high MFI values (in the range of > 15000) of DSAs as assessed by the L-SA assay [21–23].

Complement-binding anti-HLA antibodies are indeed rare in samples presenting with low MFI values in the L-SA assays. In our own research, only 3.9% and 3.2% of L-C1q-positive results were observed in classes I and II, respectively, for L-SA assays yielding results in the range of 500 to 7000 MFI. However, any attempt to establish the L-SA cut-off level even at values as low as those mentioned above remains controversial because in individual cases, positive results of L-C1q assays were observed at MFI values as low as 500. The median L-SA MFI values for anti-class I and anti-class II-immunized patients with simultaneous negative results of L-C1q assays were 3559 MFI and 5100, respectively [5].

The positive results of L-C1q assays at low L-SA MFI values are explained by the results obtained in the dilution series. The correlation between the L-SA MFI values and the L-C1q status was poor for the native sera (R = 0.248). Following a 1:16 dilution of the native serum, the correlation increased to R = 0.817. This correlation was even better than that obtained when the serum was pre-treated with EDTA before the L-SA assay (R = 0.658).

Correlations between L-C1q(+) results and individual IgG subclasses as determined in L-SA assays were also compared. The highest correlation was observed for IgG1 (R = 0.796) and IgG3 (R = 0.758). The most prevalent IgG subclasses included IgG1 (95.3%), IgG2 (54.7%), IgG3 (13.8%), and IgG4 (13.0%). Interestingly, failure to determine the IgG subclass was reported in 21.2% of cases. This was usually the case for samples with low MFI values (2774.7 ± 2457.1) [24].

The L-C1q test is capable of detecting lytic antibodies only at high serum titers which facilitate the formation of the IgG-C1q hexamers; hence the high correlation between the high MFI values in the L-SA assay and the presence of L-C1q(+) antibodies.

One can be highly confident that L-C1q(+) DSAs present a very high burden for the transplant and prognosticate transplant failure. The relationship has been confirmed by the results of a multicenter cooperative study conducted in a population of 5991 transplant recipients. Lytic anti-HLA antibodies are closely associated with the risk of antibody-mediated rejection (AMR) (HR 3.75) and transplant loss (HR = 3.09), and hence the parameter can be considered useful in evaluation of candidates for transplantation [25]. Another meta-analysis of 26 studies and 1337 transplants was published this year and compared the post-transplantation course in 485 L-C1q(+) DSA and 850 L-C1q(−) DSA recipients. In C1q(+) DSA recipients, the relative risks of antibody-mediated rejection, loss of transplant, and death amounted to RR = 2.09, RR = 2.4, and RR = 3.13, respectively. No correlation was observed between the presence of lytic antibodies and the risk of delayed graft function (DGF) or rejection of any type other than selective AMR [26].

One should keep in mind that the presence of C1q(−) DSA antibodies is not indicative of the absence of risk. Pre-transplantation C1q(−) DSAs may become C1q(+) DSAs following the transplantation. This may be due either to class switching or a change in the ratio of antibodies (reaction stoichiometry) facilitating the detection of complement-binding IgG subclasses [27–29].

Despite the controversies related to the technical aspects of detection of C1q-binding anti-HLA antibodies, it is clear that their correlation with risk parameters is higher than in the case of classic L-SA assays. The decision to include the L-C1q assay within the national

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We are planning to replace vXM based on the DSA cut-off value of > 5000 MFI with the L-C1q(+) status as the exclusion criterion. DSA > 5000 MFI/DSA C1q(–) recipients shall be qualified for biological assays (CM-CDC, cFCXM) and, in the event of a negative result, may be eligible for transplantation with immunosuppression protocol and restrictive monitoring as adequate for high-risk patients.

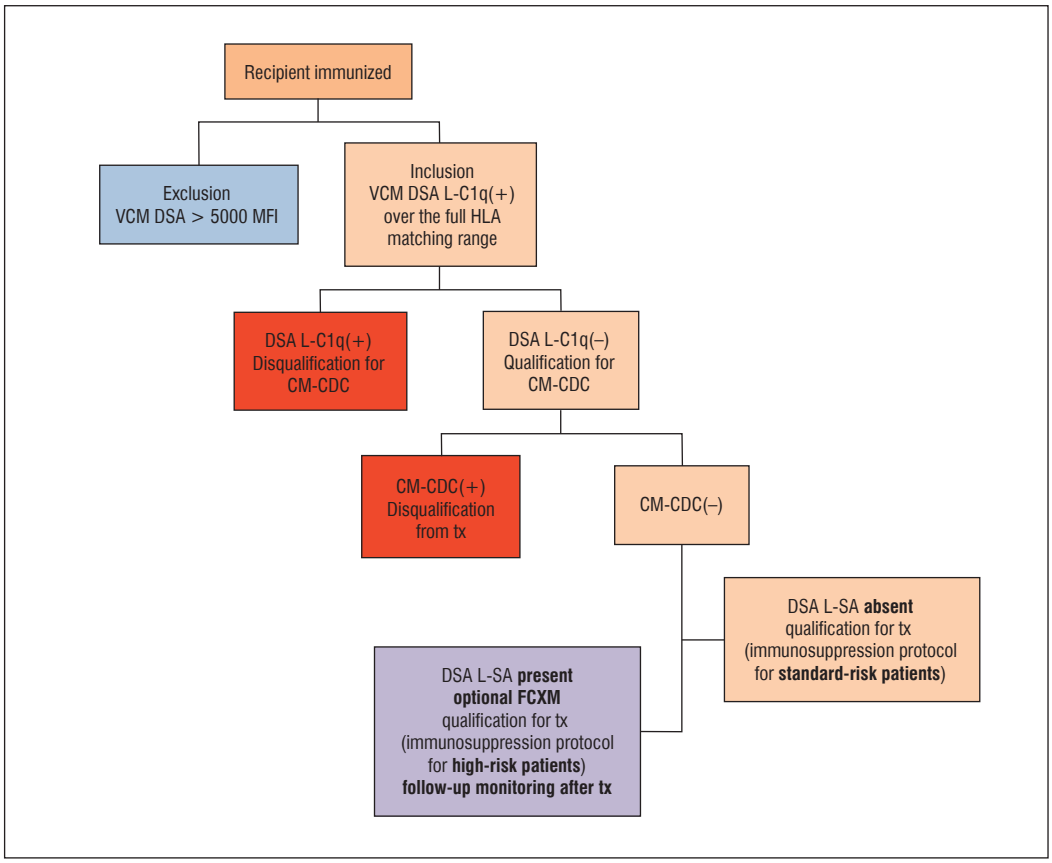

Figure 1. Management regimen including the presence of donor-specific antibodies

CM-CDC — complement dependent cytotoxicity crossmatch; DSA — donor specific antibody; FCXM — flow cytometry crossmatch; L-C1q — Luminex C1q assay; L-SA — Luminex Single Antigen assay; vXM — virtual cross match

algorithm of routine pre-transplantation risk assessment shall contribute to improved precision of donor matching and increase the recipient's chance for successful transplantation. Currently, with the sources of recipient HLA immunization being unknown, the deceased donor's DSA of > 5000 MFI excludes the recipient from further qualification procedure. We are planning to replace vXM-based on the DSA cut-off value of > 5000 MFI with the L-C1q(+) status as the exclusion criterion. DSA > 5000 MFI/DSA C1q(–) recipients shall be qualified for biological assays (CM-CDC, cFCXM) and, in the event of a negative result, may be eligible for transplantation with the immunosuppression protocol and restrictive monitoring as adequate for high-risk patients (Fig. 1). In light of numerous uncertainties, the need for follow-up studies and introducing modifications to the algorithm in line with the obtained results must be highlighted [29, 30].

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