Principles of virtual crossmatch testing for kidney transplantation

Bhaskaran, M.C.; Heidt, S.; Muthukumar, T.

KI REPORTS
KIReports.org

Principles of Virtual Crossmatch Testing for Kidney Transplantation

Madhu C. Bhaskaran[1,2], Sebastiaan Heidt[3] and Thangamani Muthukumar[4,5]

[1]Division of Kidney Diseases and Hypertension, Department of Medicine, Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Great Neck, New York, USA; [2]Kidney Transplant Program, Northwell Health, Manhasset, New York, USA; [3]Department of Immunology, Eurotransplant Reference Laboratory, Leiden University Medical Center, Leiden, The Netherlands; [4]Division of Nephrology and Hypertension, Department of Medicine, Weill Cornell Medical College New York, New York, USA; and [5]Department of Transplantation Medicine, New York Presbyterian Hospital, New York, New York, USA

Human leukocyte antigens (HLAs) are the primary determinants of alloimmunity. A crossmatch test is a test that determines the immunologic risk of a recipient with a potential donor by ensuring that there are no transplant-relevant circulating antibodies in the recipient directed against donor antigens. Physical crossmatch (PXM) tests, such as complement-dependent cytotoxicity crossmatch (CDCXM) and flow cytometry crossmatch (FCXM), require mixing of patient serum and donor cells, are labor intensive, and are logistically challenging. Virtual crossmatch (VXM) test assesses immunologic compatibility between recipient and potential donor by analyzing the results of 2 independently done physical laboratory tests—patient anti-HLA antibody and donor HLA typing. The goal of VXM is pretransplant risk stratification—though there is no consensus on whether such risk assessment involves predicting the PXM result or the posttransplant outcome. Although the concept of VXM is not new, the advent of solid-phase assays for detecting circulating antibodies in the recipient directed against individual HLA and DNA-based methods for typing donor HLA specificities at a higher resolution makes the routine use of VXM a reality. Accordingly, VXM may be applied at different scenarios—both for sensitized and nonsensitized patients. Implementation of VXM-based approach has resulted in statistically significant reduction in cold ischemia time without an increase in hyperacute rejection episodes. Though there are considerable challenges, VXM is expected to be used more often in the future, depending on the transplant center's tolerance of immunologic risk.

Kidney Int Rep (2022) **7,** 1179–1188; https://doi.org/10.1016/j.ekir.2022.03.006
KEYWORDS: calculated panel reactive antibody; donor specific antibody; human leukocyte antigen typing; kidney allocation; single antigen bead assay; unacceptable antigen

The American Society for Histocompatibility and Immunogenetics defines VXM test as an assessment of immunologic compatibility based on patient's alloantibody profile compared with donor's histocompatibility antigens.[1] Conventional PXM tests, such as CDCXM and FCXM, require mixing of patient serum and donor cells, are labor intensive, and remain logistically challenging. The VXM test uses the results of 2 independently done physical laboratory tests and does not involve mixing of serum and cells. Instead, immune compatibility is assessed by analyzing results of donor HLA typing and patient antibodies against HLA.[2] The purpose of this review is to describe the principles of the VXM test for kidney transplantation.

What Is a Crossmatch Test?

A test to determine the immunologic risk of a recipient with a potential donor by ensuring that there are no transplant-relevant circulating antibodies in the recipient directed against donor antigens. Crossmatch test was initially described for blood transfusion—the first recorded serologic crossmatch was done in 1908.[3] The first successful kidney transplantation in 1954 was between identical twins, and hence compatibility was not an issue. In 1964, Paul Terasaki postulated that preformed allogeneic antibodies present in a recipient were responsible for immediate kidney allograft failure and suggested that lymphocyte cytotoxicity may be used to detect and match transplantation antigens.[4]

Correspondence: Thangamani Muthukumar, Division of Nephrology and Hypertension, Department of Medicine, Weill Cornell Medical College, 525 East 68 Street, Box 3, New York, New York 10065, USA. E-mail: mut9002@med.cornell.edu

Received 30 November 2021; revised 14 February 2022; accepted 7 March 2022; published online 15 March 2022

How Is the HLA Phenotype of an Individual Determined?

HLAs are cell surface glycoproteins that are the main determinants of alloimmunity—the immune response to nonself antigens from members of the same species. There are >20,000 HLA class I alleles, expressed on all nucleated cells, and >8000 HLA class II alleles, expressed on (i) professional antigen-presenting cells (B cells, dendritic cells, and macrophages) and (ii) endothelial cells (http://hla.alleles.org/). There are 3 major HLA class I genes: HLA-A, HLA-B, and HLA-C. There are also 3 major HLA class II genes—HLA-DP (α-chain encoded by HLA-DPA1 locus and β-chain by HLA-DPB1), HLA-DQ (α-chain by HLA-DQA1 and β-chain by HLA-DQB1), and HLA-DR (α-chain by HLA-DRA and 4 β-chains by HLA-DRB1, DRB3, DRB4, and DRB5 loci [only 3 possible for an individual, maximum 2 of DRB1 can be associated with maximum 2 of DRB3/4/5]). Despite the large number of alleles, only a third of them have been reported commonly in unrelated individuals.[5] Hence, attempts were made to catalogue the alleles; the common and well-documented alleles, established by the American Society for Histocompatibility and Immunogenetics, are those HLA alleles for which population frequencies are well known.[6] This catalogue is widely used for resolving HLA typing ambiguities.

Historically, serologic typing was used to define the HLA phenotype by individually mixing a person's lymphocytes with several sera containing well-defined HLA antibody specificities. Currently, HLA phenotype is determined by DNA typing, by either polymerase chain reaction (e.g., reverse sequence-specific oligonucleotides, sequence-specific primers, and sequencing-based typing) or next-generation sequencing.

In the HLA nomenclature developed in 2010, each HLA allele name has a unique number corresponding to up to 4 sets of digits (called fields) separated by colons.[7] For example, in HLA-A*02:101:01:02N, field 1 corresponds to serologically defined HLA protein (HLA-A2 by serology corresponds to HLA-A*02 by DNA typing). Field 2 is the HLA allele that encodes the same protein sequence within the antigen-binding site (HLA-A*02:101 and HLA-A*02:102 are different proteins but cannot be differentiated by serologic typing). Field 3 represents DNA substitutions in the coding region but a synonymous polymorphism that does not change the protein (HLA-A*02:101:01 and HLA-A*02:101:02 have different DNA sequences but same amino acids in the protein). Field 4 is a polymorphism in the protein-noncoding region. The alphabet "N" is an optional suffix added to an allele to indicate its absence of expression.

Low-resolution DNA typing corresponds to serologically defined types and is at the level of field 1 resolution. High-resolution DNA typing defines DNA sequence of antigen-binding site and is at the level of field 2 resolution. Allelic resolution is up to the level of field 4 resolution. An allele is a unique nucleotide sequence for a gene as defined by the use of all of the digits in a current allele name.[8]

For deceased donor kidney transplantation, owing to urgency and cost constraints, most laboratories report HLA at field 1 resolution only. However, based on the linkage disequilibrium between HLA alleles of different loci, untyped HLA data may be estimated by imputation tools (impute missing untyped genotypes from neighboring typed single-nucleotide polymorphisms using a reference panel) or inference tools (infer field 2 genotypes from field 1 results based on the observed HLA allelic frequencies and haplotypes in different ethnic populations).[9]

The part of an antigen recognized by the antibody is called an epitope. Historically, it was appreciated that antibodies developed after exposure to a single allo-HLA reacted with certain other allo-HLAs as well. These "cross-reactive groups" share epitopes. Within this epitope, a central cluster of 2 to 5 amino acids (functional epitopes or eplets) determines the binding specificity of an antibody. If a donor and recipient HLA share an epitope, then that epitope will not be recognized as foreign and therefore will not provoke an antibody response.

What Are These Circulating Alloantibodies and How Are They Detected?

Antibodies of importance in kidney transplantation—in addition to ABO blood group antibodies—are those that are directed against HLA. Historically, the microlymphocytotoxicity assay or CDC assay,[10] modified for antibody screening by using a panel of HLA-typed cells and testing each individual's serum against this panel, was used for detecting anti-HLA antibodies.

An assay in which at least 1 molecule under analysis (an antigen or an antibody) is bound to a solid surface such as a microsphere and the other reactants are free in solution is called a solid-phase assay. Such an assay platform commonly used for detection of anti-HLA antibodies is the one developed by Luminex Corporation. This test is a bead-based multiplexed immunoassay that combines proprietary microsphere technology with fluidics, optics, and digital signal processing to detect several hundred targets simultaneously within a single run and works on the principles of flow cytometry.

In single-antigen bead (SAB) assay, a bead set is used for each serum sample. Each HLA class I and HLA class

II bead set are a mixture of multiple uniquely colored individual beads—each coated with a single purified HLA protein at an allelic level (e.g., B*44:02 and B*44:03 are separate beads) produced by recombinant technology. Bead sets cover the most prevalent HLA phenotype in the general—predominantly Caucasian—population. Test serum and beads are incubated allowing HLA-specific antibodies present in the serum bind to the target HLA on the beads. Fluorescent dye-labeled antihuman IgG is then added. The fluorescent emission of this dye and the individual bead's dye signature are simultaneously detected using 2 lasers. The SAB output is reported as a unitless mean (or median) fluorescence intensity (MFI) value—a semi-quantitative assessment that does not reflect accurately the concentration or titer of the antibody but gives bead's relative fluorescence without reference to a standard.[9]

Why Do Some Individuals Develop Anti-HLA Antibodies?

Antibodies against HLA typically develop after sensitizing events, such as blood transfusion, pregnancy, or organ transplantation. Unlike antibodies against blood group antigens, humans were thought not to have naturally occurring anti-HLA antibodies. However, with the advent of SAB assays, the existence of antibodies to HLA in the serum of healthy males not immunized to HLA has been described.[11] Such antibodies are probably due to cross-reactive epitopes found in microorganisms, ingested proteins, and allergens and may not be directed against an intact HLA. Because HLA coated in SAB assays are denatured molecules (beads by 1 of the 2 vendors), these can expose cryptic neoantigens due to alteration in the tertiary structure of HLA.[12] Acid treatment of beads may further denature HLA, expose more cryptic antigens, and increase the MFI value of antibodies against denatured HLA.[13] *In vivo*, such antigens are not normally accessible to these antibodies. Natural antibodies have no clinical impact in kidney transplantation.[14] Nonetheless, they may be perceived as pathogenic, influence risk stratification, and interfere with organ allocation and transplantation decisions. Besides such natural antibodies, proinflammatory events such as surgery, trauma, infections, and vaccinations have been associated with an increase in anti-HLA antibodies, likely owing to nonspecific activation of HLA-cognizant memory B cells.[15,16]

What Are Donor-Specific Antibodies?

When the SAB assays detect the presence of antibodies that are directed against donor HLA, then these are called donor-specific antibodies (DSAs). A patient being evaluated for a kidney transplantation may have circulating antibodies against HLA-A2. If this patient's prospective donor has HLA-A2, then this antibody against HLA-A2 is called a DSA. Though used generically, the term DSA—as detected in most laboratories—implies anti-HLA IgG antibodies.

What Is a CDCXM Test?

The CDCXM test is a test that detects the functional potential of complement-fixing antibodies in the recipient's circulation which can immediately bind to and react against the donor kidney, providing direct evidence for the presence of likely pathogenic (i.e., cytotoxic) alloantibodies that can result in hyperacute rejection. It is an *in vitro* test in which recipient serum is mixed with donor lymphocytes or donor T or B cells obtained from either the peripheral blood of living donors or the lymph nodes, spleen, or peripheral blood of deceased donors. Complement (usually from rabbit serum) is added to the mixture. The readout of this test is the detection of donor cell death. Results are usually expressed on a semiquantitative scale. Worldwide, different scoring systems are used.

What Is a FCXM Test?

The FCXM test is a test that identifies the antibodies directed at cell surface antigens. Whether the detected antibodies are pathogenic or not cannot be predicted with high confidence. Introduced in early 1980s, this *in vitro* test involves mixing of recipient serum and donor lymphocytes. Alloantibodies, if present, bind to the cells. Donor T and B lymphocytes need not be separated—cell lineage-specific antibodies are added to distinguish T cells and B cells. Fluorescein-labeled antihuman IgG polyclonal antibody is then added to detect the bound alloantibodies of IgG isotype. Fluorescence intensity is assessed as median channel fluorescence or molecules of equivalent soluble fluorochrome. The readout of this test is the change in fluorescence intensity. If alloantibodies are bound to the cells, there will be a higher fluorescence intensity compared with the negative control serum. A predetermined value for this increase in intensity is classified as a positive crossmatch. Whereas the CDCXM uses a functional readout of cell lysis, in the FCXM, only the binding of HLA-specific antibodies is detected, regardless of their complement fixing or pathogenic potential.

Is Transplantation Contraindicated When a Crossmatch Test Result Is Positive?

In a crossmatch test, donor lymphocytes are surrogate for donor kidney cells. Thus, a positive crossmatch result implies that circulating alloantibodies are present

in the patient with the potential to kill (CDCXM) or bind (FCXM) donor kidney cells. T cells express HLA class I and B cells express both HLA class I and class II. Positive T cell crossmatch result implies the likely presence of antibodies against HLA class I, and a positive B cell crossmatch result implies the likely presence of antibodies against both class I and class II. When combined, a negative T cell crossmatch result and a positive B cell crossmatch result suggest the presence of class II-reactive antibodies only. Rarely, non-HLA antibodies lead to a positive crossmatch result. In the seminal article of 1969, Patel and Terasaki[17] found that 24 of 30 (80%) kidneys transplanted across a positive CDCXM result failed immediately, in contrast to 8 of the 195 (4%) kidneys transplanted across a negative crossmatch result. Accordingly, a positive T cell CDCXM result is considered an absolute contraindication for transplantation because of its association with hyperacute/accelerated rejection. A positive B cell CDCXM result or a positive FCXM result increases the risk to an intermediate level and may need desensitization therapy prior to transplant but is not considered an absolute contraindication for transplantation, although regional differences in policies exist.

What Is Calculated Panel Reactive Antibody?

Panel reactive antibody (PRA) is a test that identifies sensitized patients and estimate their likelihood of finding a crossmatch-compatible donor.[18] Historically, patient's serum was tested against lymphocytes obtained from a panel of about 40 to 100 blood donors who represent the potential HLA makeup for a donor from that geographic area. The percentage of positive reactions gave rise to the percentage PRA.

The calculated PRA (CPRA), mandated in the United States since 2009 to assess immune sensitization status, is a value that is based on the unacceptable antigens. Other organ allocation organizations have incorporated identical, though differently named values, such as virtual PRA in the Eurotransplant program (allocates organs in Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands, and Slovenia[12]) and calculated reaction frequency in the United Kingdom. When a patient has antibodies against one or more antigens (HLA-A, B, C, DR, and DQ) that are present above a threshold MFI (USA) and/or proved to be cytotoxic by CDC assay (Eurotransplant), then those antigens are reported as unacceptable for the patient. During allocation, kidneys expressing those HLA are not offered to the patient.[19]

The CPRA algorithm (https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/) uses the unacceptable antigen list of a patient to derive a 15-digit decimal between 0 and 1 based on the HLA frequencies of kidney donors in the USA. The CPRA value is the population frequency of organ donors expressing one or more of those unacceptable antigens. The 15-digit value is stored, but rounded to nearest hundredth (2-digit decimal), multiplied by 100, and expressed as a percentage that is used for organ allocation. For example, if a patient has a CPRA of 4%, then that person is expected to be able to safely accept an organ (and not experience immediate rejection) from approximately 96% of organ donors from the population.

Among sensitized patients, the distribution of CPRA is not uniform but peaks at 100%. However, as CPRA is a rounded integer, within the 100% designation, the probability of matching varies according to the unrounded value.[20] When to call an HLA as unacceptable for a given patient (at what MFI value of a HLA antibody in the SAB assay should that HLA be considered unacceptable) is left to the discretion of individual transplant program and is a reflection of their willingness to assume the risks associated with anti-HLA antibodies.[21]

A high-percentage PRA implied a high probability of a positive crossmatch result. However, because CPRA is based on unacceptable antigens, kidneys with those antigens are not offered for a patient. Thus, an actual offer for a patient with high CPRA—taking the unacceptable antigens into account—implies high probability of a negative crossmatch result.[10] Importantly, although the broadness of HLA sensitization is determined by the CPRA and influences organ allocation, in terms of immunologic risk for the recipient, it is the donor specificity—not the broadness of sensitization—that is associated with allograft outcome.[22] About 10% of waitlisted patients at large US transplant centers are highly (≥98%) sensitized.[23]

In December 2014, a new kidney allocation system was introduced in the United States. Currently, there are >90,000 patients waiting in the USA to receive a kidney. The number of match runs required to have a 95% chance of finding an acceptable donor for a patient with 10% CPRA is 2 but increases to 300 for 99% CPRA.[20] Accordingly, allocation is based on a sliding scale point system—additional points for sensitization begin at 20% CPRA and increase exponentially as it approaches 100%. Patients with CPRA >98% also receive regional and national priorities for allocation, expanding the donor pool. After the introduction of the new kidney allocation system, there has been a significant increase in the transplantation of patients with CRPA of ≥98%.[24,25]

What Is an Acceptable Mismatch?

Similar to the "unacceptable antigen" concept in the United States, in the Eurotransplant program, a concept

called "acceptable mismatch" is used for transplanting highly sensitized patients.[12] The premise of the "Acceptable Mismatch program" is that by actively defining the acceptable antigens for an individual (antigens against which the individual has never formed antibodies), a negative PXM can be predicted. The chance for highly sensitized patients to be transplanted is increased by the addition of the acceptable antigens to their HLA phenotype, thereby creating an "extended" HLA phenotype, based on which allocation takes place. This strategy has been found to be cost effective and has resulted in favorable rejection rates and graft survival.[26,27]

How Is the Result of VXM Test Reported?

Unlike CDCXM and FCXM, in VXM, the immunologic compatibility between a patient and a donor is determined *in silico*. Although VXM is a risk assessment tool, there is currently no consensus on the goal of VXM—whether the risk assessment involves predicting the result of a PXM or the posttransplant outcome.[28] For nonsensitized patients who do not have circulating antibodies, PXM result against any prospective donor can be assumed to be negative. Such a scenario is applicable for the majority (>75%) of waitlisted patients. For those who have antibodies by SAB assay, the risk stratification varies according to the laboratory's goal of doing a VXM and may include multiple MFI cutoff values to call an antibody as present or unacceptable or to predict FCXM or CDCXM results. Accordingly, the output of VXM could be reported as a dichotomous positive or negative or as a probability value and has not been standardized.

Is VXM More Sensitive Than PXM?

In the SAB test, when to call an antibody as present/positive or absent/negative depends on one of the following 3 approaches: (i) statistical (fluorescence level above background), (ii) practical (correlate with PXM result), and (iii) clinical (correlate with rejection or graft failure).[29] MFI values are routinely used as an assessment of antibody strength despite the fact that it is a relative fluorescence and can be affected by many variables; also, fluorescence intensity has only a weak relationship with the titer of specific antibody in the tested serum.[30,31] Cutoff values, thus, are different among laboratories and have not been standardized. A Clinical Trials in Organ Transplantation study concluded that MFI value of 1000 to 1500 yielded a high level of agreement among HLA laboratories to determine the presence or absence of an antibody.[32] A Dutch multicenter study has proposed a signal-to-background ratio, rather than an MFI cutoff, for pretransplant risk stratification.[31] As most laboratories use a statistical approach, the SAB test can detect DSA even when the PXM result is negative. Thus, if laboratories report VXM as positive whenever DSA is positive, then VXM is potentially more sensitive than PXM to detect the presence of DSA. Importantly, the higher sensitivity could result in a group of patients who have a positive VXM result but a negative PXM result. Such a scenario could mean a low burden of DSA or non–complement-fixing DSA (FCXM+ and CDCXM−).

Technical issues related to the assay and a low threshold for calling an antibody as present (over-calling) could result in a false-positive DSA result. Patient's sensitization history, knowledge about the cross-reactive groups, epitope analysis, CDC screening, and refinements to the SAB assay such as serial dilution of the serum are some of the strategies used by laboratories to determine the specificity of antibodies.[33] Thus, depending on the unacceptable antigen listing strategy, a positive DSA/VXM result need not always mean the presence of a clinically significant anti-HLA antibody. The expression of HLA on the allograft endothelium, the avidity of eplet-antibody interaction (electrostatic potential of an amino acid polymorphism within an eplet influences the avidity of the antigen-antibody complex), complement fixing ability, and the IgG subclass of the antibody are some of the determinants that are thought to impact the pathogenicity of DSA.[33]

How Can VXM Be Applied in Clinical Transplantation?

Application of VXM is possible both at the time of organ allocation and after organ allocation. At organ allocation, the process of listing unacceptable antigens or acceptable mismatches for a given patient and organ allocation based on that listing is equivalent to a negative VXM result—the antibody profile is used for predicting compatibility with prospective donors.[34]

After allocation, at the transplant center level, VXM may be applied both for nonsensitized patients and sensitized patients (Figure 1). Several transplant centers have reported their success with proceeding to transplant based on VXM results and eliminating pretransplant PXM.[35–42] The need for pretransplant PXM increases cold ischemia time—the time from clamping of the donor renal artery to restoration of blood flow after transplantation. Cold ischemia is associated with increased risk for delayed graft function, organ discards, and graft failure.[43–45] A recent analysis of US registry data identified 9632 kidney transplants between 2011 and 2018 using VXM and 71,839 using PXM.[42] Cold ischemia time was significantly lower in the VXM group (mean 15.0 hours) compared with the

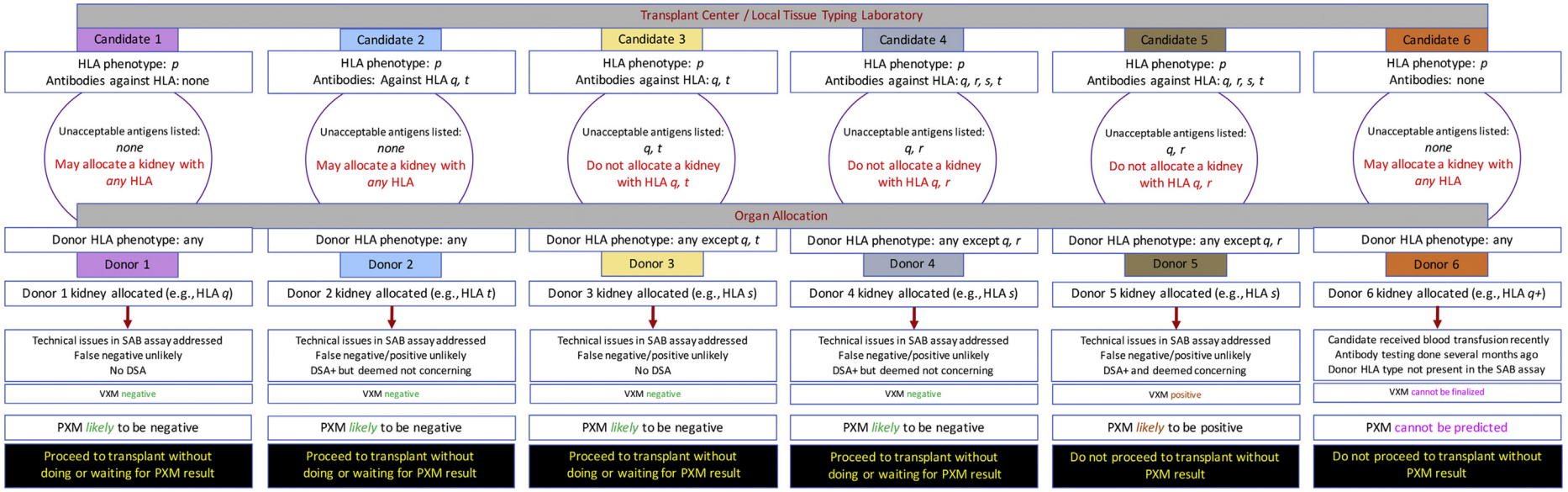

Figure 1. Potential applications of virtual crossmatch test at the recipient center for kidney transplantation. The application of VXM may be envisioned for different scenarios. Figure depicts 6 potential candidates awaiting kidney transplantation. HLA *p*, *q*, *r*, *s*, and *t*, are representative examples reflecting the spectrum of HLA phenotype in the community. Candidate 1 is not sensitized and has no circulating antibodies against any HLA; VXM result is reported as negative and this candidate's PXM result is likely to be negative against any donor. Candidate 2 has HLA-specific antibodies but does not meet the individual center's criteria for listing those HLA as unacceptable antigens. Once an organ is allocated for this candidate, though this individual has DSA, after careful analysis of the donor HLA and recipient antibody profiles, the laboratory may report the VXM result as negative and the center may decide to proceed with the transplant. Candidate 3 is highly sensitized (e.g., CPRA 99%). Based on the candidate's reported unacceptable antigens, organ allocation is personalized. Accordingly, an offer for this individual—after taking the reported unacceptable antigens into consideration—should mean a high probability of a negative PXM result, provided there are no other DSA of concern. Candidates 4 and 5 are identical in terms of their HLA phenotype, antibody profile, and listing of unacceptable antigens. After organ allocation, the local laboratory does a careful analysis of donor HLA and recipient antibody profile and may report the VXM result as negative for candidate 4 and as positive for candidate 5. Accordingly, the center may procced with the transplant for the former but may request a PXM test and delay the transplant pending the results of PXM for the latter. In the United States, when to call an HLA as unacceptable for a given patient is left to the discretion of the transplant center. Thus, for an individual patient, there may be a discrepancy in VXM results during allocation (kidney offer taking into account the unacceptable antigens are equivalent to a negative VXM result) versus after allocation (VXM result may be positive taking into account other DSA that were not reported as unacceptable). It may not be possible to finalize VXM result under certain circumstances. Candidate 6 is such an example, where a kidney is offered from a donor who has a HLA phenotype that is not represented in the SAB assay. Because HLA *q*+ antigens of donor 6 are not present in the beads in the SAB assay, antibodies directed against HLA *q*+ in candidate 6—if present—would not be detected in the SAB assay. Accordingly, failure to detect antibodies in candidate 6 directed against HLA *q*+ is not a proof of absence of that antibody in circulation. Also, when DSA is positive, decision to proceed to transplant based on VXM results depends on the transplant center's willingness to assume the long-term risks associated with antibodies against HLA. Nevertheless, option to do a PXM for ambiguous results or for predetermined center or patient-specific criteria should always be available. Also, decisions based on patient anti-HLA antibody profile assume—currently in most laboratories—that circulating IgG antibodies against HLA are the only antibodies of relevance in transplantation. CPRA, calculated panel reactive antibody; DSA, donor- specific antibody; HLA, human leukocyte antigen; PXM, physical crossmatch; SAB, single-antigen bead; VXM, virtual crossmatch.

PXM group (mean 16.5 hours). Importantly, mortality and death-censored allograft failure were similar.

Although discussion of individual studies is beyond the scope of this review, it is useful to summarize the following 2 recent studies: (i) In a single-center study of 254 kidneys that were imported to that center between 2014 and 2017, among the 137 patients with CPRA >98%, 118 (86%) were transplanted without a pretransplant PXM. There were no hyperacute rejections, only 5 acute rejections, and no differences in graft function at 1 year between those transplanted based on a VXM and those who needed a PXM,[39] (ii) In a single-center study of 825 patients who received a kidney between 2014 and 2018, 227 transplants were done after the implementation of VXM. Although nearly a third of all patients had CPRA >80%, none had hyperacute rejection, and the results of PXM tests done after the completion of transplant were not discordant with the VXM.[40]

In March 2021, allocation policy in the United States was changed such that kidneys will be offered first to candidates listed at transplant hospitals within 250 nautical miles (288 miles/463 km) of the donor hospital and then to candidates beyond that distance. This change is likely to increase travel distance and accrued cold ischemia—using more VXM could help overcome some of the logistical challenges.[42]

VXM Appears to Be Intuitive. Why Is It Not Being Used Routinely?

The concept of using unacceptable antigens based on the identification of HLA-specific antibodies to eliminate potential donors from allocation lists is not new.[46] However, until solid-phase assays became widely available, the CDC assay that was used to determine the antibodies was largely based on HLA class I antigens and could not define all antibody specificities, especially in patients who were broadly sensitized. With the advent of DNA-based methods for typing HLA specificities at a higher resolution and the solid-phase assays for detecting antibodies against individual HLA, VXM has come to the limelight. After the implementation of kidney allocation system in the United States, there has been a steady increase in the use of VXM—in 2018, 18% of kidney transplants were done relying on a VXM.[42] However, there are several prerequisites that need to be met for the routine successful implementation of VXM.

What Are the Challenges in the Routine Implementation of VXM Testing?

For patients who do not have HLA antibodies by SAB assay, pretransplant PXM may be unnecessary, irrespective of the HLA type of the potential donor, provided there were not any recent sensitizing events in the recipient. Although this statement is intuitive and straightforward, there are multiple challenges—owing to limitations of the SAB assay—in defining the absence or presence of circulating antibodies against HLA.[28] For example, (i) the SAB assay may not contain beads for a particular specificity; (ii) SAB manufacturing issues result in lot-to-lot variability[47]; (iii) HLA structure present on the SAB assays differs between the 2 vendors that are currently available; (iv) shared epitopes—antibody targets that are shared by multiple antigens on the panel tested—may result in dilution of the antibodies that bind to each antigen[29]; and (v) inhibitory factors present in the patient serum—such as complement C1q—may interfere with antibody detection (prozone effect) and require pretreatment of serum with heat, dithiothreitol, or ethylenediamine tetra-acetic acid, or dilution of the serum.[48] All these could result in false-negative result (DSA present but reported as negative in the SAB assay). Thus, the failure to detect an antibody on a SAB assay is not a proof of absence of that antibody in circulation.

In addition, the density of antigen on the beads, the fluorochrome detection antibody used, and the setup of the instrument used for SAB assay may affect the MFI value. Accepting all antibody reactivity in the SAB assay as positive and clinically relevant carries the risk of false assignment of unacceptable antigens and diminishing a patient's chance of receiving an organ offer. Importantly, SAB assay uses synthetic HLA on beads as the target and may not reflect the cellular profile—for example, cell surface expression of HLA-C is lower than HLA-A and -B,[49] but this is not reflected on the beads. Also, beads are coated with finite number of antigens and hence are saturable. Therefore, for example, in Eurotransplant, the CDC assay is used in conjunction with SAB assays. In addition, a patient's recent infection and immunization history must be considered when determining whether an antibody reactivity should lead to listing that antigen as unacceptable.

To accomplish the goal of assessing immunologic compatibility between a patient and a donor, VXM relies on the precise and complete donor HLA typing.[28] There are ambiguities associated with HLA typing. Ideally, prospective donors must be HLA typed at the resolution, at least, of field 2. Unambiguous second field typing in the time frame of a deceased donor procedure is not yet routine but feasible.[50] Currently, although laboratories in the United States are required to determine HLA phenotype by DNA typing, they are not mandated to report it at field 2 resolution. For example, donor HLA phenotype may be reported at field 1 resolution as B44. If a patient has antibody

against B*44:02 and not B*44:03 by SAB assay, then without knowing the donor HLA at the level of field 2 resolution (is the donor HLA-B*44:02 or B*44:03), it is not possible to determine whether the antibody against B*44:02 is donor specific or not.[28] Thus, although sensitive SAB assays are able to identify antibodies in the prospective recipient against HLA at field 2 resolution (when listing a patient's unacceptable antigen, UNet [organ transplant web platform in the United States] allows reporting of HLA at field 2 rather than field 1), because prospective donor genotyping is mostly reported at field 1 resolution, this creates a challenge in the interpretation of HLA compatibility and in decisions on organ allocation.[9] Eurotransplant, accordingly, will implement reporting of ambiguous field 2 typing for VXM that will be filtered against common and well-documented alleles and used for VXM, until unambiguous field 2 typing techniques for deceased donor transplants are routinely available.

There are regulatory and cost considerations that impede the quick adaption of VXM. Each laboratory is required to perform a crossmatch according to the terms specified in the written agreement between the laboratory and the organ procurement organization or the transplant program. Also, how often should patients waitlisted for a transplant be screened for the presence of circulating antibodies is program specific (most laboratories do not use serum samples dated no longer than 1–3 months prior to the expected transplant date) and is a balance between cost of screening and the need for updated antibody information.

So, What Is the Future?

Interesting debates on this topic are ongoing among experts. Some have suggested routine implementation of VXM, at least in the short term for donors of Caucasoid origin—provided a quick assay for high-resolution HLA typing is readily available for deceased donors—and have proposed that PXM should only be considered for highly sensitized patients when there is ambiguity in donor typing or if the donor allele is not present on the single antigen bead.[51] Others have urged caution given the limitations of our current assays and provide compelling argument to develop algorithms to assign strength to antibodies, at the minimum, to distinguish unacceptable antigens from antigens against which antibody is present but the program is willing to consider offers from donors with these antigens.[28] Thus, continuous education of all stakeholders and standardization of HLA laboratory practices are needed.[2] With a switch to distance-based kidney allocation in the United States in 2021, it is likely that VXM will be used more often in the future—especially for the first-time kidney transplant male recipient with no IgG anti-HLA antibodies and no recent sensitizing events. However, PXM will still be needed and used, albeit judiciously, depending on the individual center's tolerance of immunologic risk.[52]

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

The authors (MCB and TM) thank the following: (i) Manikkam Suthanthiran, MD, Stanton Griffis Distinguished Professor of Medicine, Chief of Nephrology and Hypertension, Chief of Transplantation Medicine, New York Presbyterian Hospital, Weill Cornell Medical Center, New York, NY, Founder-Director of the Immunogenetics & Transplantation Laboratory, The Rogosin Institute, New York, NY, for his guidance and support; (ii) Vijay K. Sharma, PhD, Associate Research Professor of Molecular Biology, Division of Nephrology and Hypertension, Weill Cornell Medical Center, Director of the Immunogenetics & Transplantation Laboratory, The Rogosin Institute, New York, NY, for his help; and (iii) Rex Friedlander, CHS, Manager of the Immunogenetics & Transplantation Laboratory, The Rogosin Institute, New York, NY, for his help.

REFERENCES

1. Bray RA. The acceptability and application of virtual crossmatching in lieu of serologic crossmatching for transplantation. Virtual Crossmatch Work Group Report. Centers for Disease Control and Prevention. Published 2014. Accessed April 1, 2022. https://www.cdc.gov/cliac/docs/addenda/cliac1114/8_BRAY_Virtual_Crossmatch_Workgroup_Report_Nov-2014.pdf
2. Morris AB, Sullivan HC, Krummey SM, et al. Out with the old, in with the new: virtual versus physical crossmatching in the modern era. *HLA*. 2019;94:471–481. https://doi.org/10.1111/tan.13693
3. Oberman HA. The crossmatch. A brief historical perspective. *Transfusion*. 1981;21:645–651. https://doi.org/10.1046/j.1537-2995.1981.21682085750.x
4. Terasaki PI, Marchiori TH, Starzl TE. Sero-typing of human lymphocyte antigens: preliminary trials on long-term kidney homograft survivors. In: Russell PS, Winn HJ, eds. *Histocompatibility Testing*. Washington, DC: National Academy of Sciences, National Research Council; 1965:83–95.
5. Gonzalez-Galarza FF, Mack SJ, Hollenbach J, et al. 16 (th) IHIW: extending the number of resources and bioinformatics analysis for the investigation of HLA rare alleles. *Int J Immunogenet*. 2013;40:60–65. https://doi.org/10.1111/iji.12030
6. Mack SJ, Cano P, Hollenbach JA, et al. Common and well-documented HLA alleles: 2012 update to the CWD

catalogue. *Tissue Antigens*. 2013;81:194–203. https://doi.org/10.1111/tan.12093

7. Marsh SG, Albert ED, Bodmer WF, et al. Nomenclature for factors of the HLA system, 2010. *Tissue Antigens*. 2010;75:291–455. https://doi.org/10.1111/j.1399-0039.2010.01466.x
8. Nunes E, Heslop H, Fernandez-Vina M, et al. Definitions of histocompatibility typing terms. *Blood*. 2011;118:e180–e183. https://doi.org/10.1182/blood-2011-05-353490
9. Senev A, Emonds MP, Van Sandt V, et al. Clinical importance of extended second field high-resolution HLA genotyping for kidney transplantation. *Am J Transplant*. 2020;20:3367–3378. https://doi.org/10.1111/ajt.15938
10. Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxins. *Nature*. 1964;204:998–1000. https://doi.org/10.1038/204998b0
11. Morales-Buenrostro LE, Terasaki PI, Marino-Vazquez LA, et al. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation*. 2008;86:1111–1115. https://doi.org/10.1097/TP.0b013e318186d87b
12. Tait BD. Detection of HLA antibodies in organ transplant recipients - triumphs and challenges of the solid phase bead assay. *Front Immunol*. 2016;7:570. https://doi.org/10.3389/fimmu.2016.00570
13. Chowdhry M, Agrawal S, Thakur Y, et al. Implication of a positive virtual crossmatch with negative flow crossmatch: a mind-boggler. *Asian J Transfus Sci*. 2020;14:79–82. https://doi.org/10.4103/ajts.AJTS_159_18
14. Otten HG, Verhaar MC, Borst HP, et al. The significance of pretransplant donor-specific antibodies reactive with intact or denatured human leucocyte antigen in kidney transplantation. *Clin Exp Immunol*. 2013;173:536–543. https://doi.org/10.1111/cei.12127
15. Rees L, Kim JJ. HLA sensitisation: can it be prevented? *Pediatr Nephrol*. 2015;30:577–587. https://doi.org/10.1007/s00467-014-2868-6
16. Heidt S, Feltkamp MC, Karahan GE, et al. No evidence for cross-reactivity of virus-specific antibodies with HLA alloantigens. *Transplantation*. 2018;102:1844–1849. https://doi.org/10.1097/TP.0000000000002369
17. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. *N Engl J Med*. 1969;280:735–739. https://doi.org/10.1056/NEJM196904032801401
18. Cecka JM. Calculated PRA (CPRA): the new measure of sensitization for transplant candidates. *Am J Transplant*. 2010;10:26–29. https://doi.org/10.1111/j.1600-6143.2009.02927.x
19. Leffell MS. The calculated panel reactive antibody policy: an advancement improving organ allocation. *Curr Opin Organ Transplant*. 2011;16:404–409. https://doi.org/10.1097/MOT.0b013e3283489910
20. Keith DS, Vranic GM. Approach to the highly sensitized kidney transplant candidate. *Clin J Am Soc Nephrol*. 2016;11:684–693. https://doi.org/10.2215/CJN.05930615
21. Kamoun M, Phelan D, Noreen H, et al. HLA compatibility assessment and management of highly sensitized patients under the new kidney allocation system (KAS): a 2016 status report from twelve HLA laboratories across the U.S. *Hum Immunol*. 2017;78:19–23. https://doi.org/10.1016/j.humimm.2016.10.023
22. Wehmeier C, Honger G, Cun H, et al. Donor specificity but not broadness of sensitization is associated with antibody-mediated rejection and graft loss in renal allograft recipients. *Am J Transplant*. 2017;17:2092–2102. https://doi.org/10.1111/ajt.14247
23. Parsons RF, Locke JE, Redfield RR 3rd, et al. Kidney transplantation of highly sensitized recipients under the new kidney allocation system: a reflection from five different transplant centers across the United States. *Hum Immunol*. 2017;78:30–36. https://doi.org/10.1016/j.humimm.2016.10.009
24. Jackson KR, Covarrubias K, Holscher CM, et al. The national landscape of deceased donor kidney transplantation for the highly sensitized: transplant rates, waitlist mortality, and posttransplant survival under KAS. *Am J Transplant*. 2019;19:1129–1138. https://doi.org/10.1111/ajt.15149
25. Jackson KR, Motter JD, Kernodle A, et al. How do highly sensitized patients get kidney transplants in the United States? Trends over the last decade. *Am J Transplant*. 2020;20:2101–2112. https://doi.org/10.1111/ajt.15825
26. Heidt S, Haasnoot GW, van Rood JJ, et al. Kidney allocation based on proven acceptable antigens results in superior graft survival in highly sensitized patients. *Kidney Int*. 2018;93:491–500. https://doi.org/10.1016/j.kint.2017.07.018
27. Heidt S, Haasnoot GW, Witvliet MD, et al. Allocation to highly sensitized patients based on acceptable mismatches results in low rejection rates comparable to nonsensitized patients. *Am J Transplant*. 2019;19:2926–2933. https://doi.org/10.1111/ajt.15486
28. Pinelli DF, Tambur AR. Virtual crossmatching for deceased donor transplantation: one size does not fit all. *Kidney Int*. 2020;97:659–662. https://doi.org/10.1016/j.kint.2020.02.001
29. Gebel HM, Bray RA. HLA antibody detection with solid phase assays: great expectations or expectations too great? *Am J Transplant*. 2014;14:1964–1975. https://doi.org/10.1111/ajt.12807
30. Sullivan HC, Liwski RS, Bray RA, Gebel HM. The road to HLA antibody evaluation: do not rely on MFI. *Am J Transplant*. 2017;17:1455–1461. https://doi.org/10.1111/ajt.14229
31. Wisse BW, Kamburova EG, Joosten I, et al. Toward a sensible single-antigen bead cutoff based on kidney graft survival. *Transplantation*. 2019;103:789–797. https://doi.org/10.1097/TP.0000000000002357
32. Reed EF, Rao P, Zhang Z, et al. Comprehensive assessment and standardization of solid phase multiplex-bead arrays for the detection of antibodies to HLA. *Am J Transplant*. 2013;13:1859–1870. https://doi.org/10.1111/ajt.12287
33. McCaughan J, Xu Q, Tinckam K. Detecting donor-specific antibodies: the importance of sorting the wheat from the chaff. *Hepatobiliary Surg Nutr*. 2019;8:37–52. https://doi.org/10.21037/hbsn.2019.01.01
34. Clifford Sullivan H, Krummey SM, Gebel HM, et al. Physical crossmatching vs virtual crossmatching: the end of an era? or Why give up a good thing? *Hum Immunol*. 2020;81:401–406. https://doi.org/10.1016/j.humimm.2020.06.009
35. Taylor CJ, Kosmoliaptsis V, Sharples LD, et al. Ten-year experience of selective omission of the pretransplant crossmatch test in deceased donor kidney transplantation. *Transplantation*. 2010;89:185–193. https://doi.org/10.1097/TP.0b013e3181c926f2
36. Amico P, Hirt-Minkowski P, Honger G, et al. Risk stratification by the virtual crossmatch: a prospective study in 233 renal

transplantations. *Transpl Int*. 2011;24:560–569. https://doi.org/10.1111/j.1432-2277.2011.01235.x

37. Johnson CP, Schiller JJ, Zhu YR, et al. Renal transplantation with final allocation based on the virtual crossmatch. *Am J Transplant*. 2016;16:1503–1515. https://doi.org/10.1111/ajt.13606
38. Turner D, Battle R, Akbarzad-Yousefi A, Little AM. The omission of the "wet" pre-transplant crossmatch in renal transplant centres in Scotland. *HLA*. 2019;94:3–10. https://doi.org/10.1111/tan.13558
39. Roll GR, Webber AB, Gae DH, et al. A virtual crossmatch-based strategy facilitates sharing of deceased donor kidneys for highly sensitized recipients. *Transplantation*. 2020;104:1239–1245. https://doi.org/10.1097/TP.0000000000002924
40. Rohan VS, Pilch N, Moussa O, et al. Virtual crossmatching in kidney transplantation: the wait is over. *J Am Coll Surg*. 2020;230:373–379. https://doi.org/10.1016/j.jamcollsurg.2019.12.031
41. Aslam S, Buggs J, Wyatt K, et al. The impact of virtual crossmatch on cold ischemic times and outcomes following kidney transplantation. *Ann M Surg*. 2021;87:109–113. https://doi.org/10.1177/0003134820942180
42. Puttarajappa CM, Jorgensen D, Yabes JG, et al. Trends and impact on cold ischemia time and clinical outcomes using virtual crossmatch for deceased donor kidney transplantation in the United States. *Kidney Int*. 2021;100:660–671. https://doi.org/10.1016/j.kint.2021.04.020
43. Yarlagadda SG, Coca SG, Formica RN Jr, et al. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. *Nephrol Dial Transplant*. 2009;24:1039–1047. https://doi.org/10.1093/ndt/gfn667
44. Debout A, Foucher Y, Trebern-Launay K, et al. Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation. *Kidney Int*. 2015;87:343–349. https://doi.org/10.1038/ki.2014.304
45. Peters-Sengers H, Houtzager JHE, Idu MM, et al. Impact of cold ischemia time on outcomes of deceased donor kidney transplantation: an analysis of a national registry. *Transplant Direct*. 2019;5:e448. https://doi.org/10.1097/TXD.0000000000000888
46. Delmonico FL, Fuller A, Cosimi AB, et al. New approaches to donor crossmatching and successful transplantation of highly sensitized patients. *Transplantation*. 1983;36:629–633. https://doi.org/10.1097/00007890-198336060-00007
47. Friedlander R, Putheti P, Diaz E, et al. On the detection of anti-HLA antibodies using single antigen bead Luminex assay: lot-to-lot variations in MFI. *Transplantation*. 2013;96:e24–e26. https://doi.org/10.1097/TP.0b013e31829c2481
48. Schnaidt M, Weinstock C, Jurisic M, et al. HLA antibody specification using single-antigen beads–a technical solution for the prozone effect. *Transplantation*. 2011;92:510–515. https://doi.org/10.1097/TP.0b013e31822872dd
49. Kaur G, Gras S, Mobbs JI, et al. Structural and regulatory diversity shape HLA-C protein expression levels. *Nat Commun*. 2017;8:15924. https://doi.org/10.1038/ncomms15924
50. De Santis D, Truong L, Martinez P, D'Orsogna L. Rapid high-resolution HLA genotyping by MinION Oxford nanopore sequencing for deceased donor organ allocation. *HLA*. 2020;96:141–162. https://doi.org/10.1111/tan.13901
51. Claas FHJ, Heidt S. Virtual crossmatching for deceased donor transplantation becomes reality. *Kidney Int*. 2020;97:657–659. https://doi.org/10.1016/j.kint.2020.01.024
52. Coates PT, Wong G. Current controversies in nephrology-how to crossmatch for transplantation? *Kidney Int*. 2020;97:662–663. https://doi.org/10.1016/j.kint.2020.02.002