REVIEW ARTICLE

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Role of crossmatch testing when Luminex-SAB is negative in renal transplantation

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ABSTRACT: The human leukocyte antigen (HLA) system plays an important role in the acceptance of renal graft. Long and better graft survival has been reported in patients with HLA-identical siblings and a nonreactive cytotoxicity assay (CDC). New methods of HLA-typing and anti-HLA antibody detection techniques such as flow cytometry, solid-phase immunoassays, or antigen bead assays have further improved the outcomes of renal transplant recipients. In the present review, the explicit details of these methodologies are discussed in detail.

KEYWORDS: renal transplant, human leukocyte antigen, HLA-typing, anti-HLA antibody

INTRODUCTION

The human leukocyte antigen (HLA) system provides a major barrier to acceptance of renal transplants. In 1954, Joseph Murray et al. carried out the first successful renal transplantation between identical twins to eliminate problems related to an immune reaction. (1). Longer survival has been reported in patients with grafts from HLA-identical siblings and a nonreactive cytotoxicity assay (CDC) compared to patients with grafts from randomly matched, deceased donors despite similar immunosuppressive treatment (2,3,4). Over the last two decades, new methods such as flow cytometry, solid-phase immunoassays, or antigen bead assays such as Luminex have improved HLA-typing and anti-HLA antibody detection (5,6,7). A combined use of these newer techniques with CDC has improved immunological risk analysis (8,9).

HLA ANTIGENS

In 1952, Jean Dausset et al. first reported of the HLA system, i.e., the human major histocompatibility complex (MHC), after they noticed different kinds of alloantibodies against leukocytes in multiple blood transfusion recipients (10). The HLA system is present on chromosome 6p. Three class I HLA molecules, i.e., HLA-A, B, and C, are expressed on all nucleated cells, and they present intracellular antigens. Six class II HLA molecules, i.e., HLA- DPA1, DPB1, DQA1, DQB1, DRA, and DRB1, are expressed only by antigen-presenting cells and lymphocytes, and they present extracellular antigens (11). By presenting antigens to lymphocytes, the HLA molecules help in differentiating self from non-self. The magnitude of HLA mismatch at the A, B, and DR loci predicts the risk of graft rejection in potential recipients (12,13,14,15). When matching donors and recipients, one should avoid mismatching rather than matching each HLA antigens because of homozygous presentation of antigens e.g., HLA-A2.

The CDCXM was first introduced by Terasaki et al. in the 1960, and since then, it has become a standard technique to detect clinically significant donor- specific HLA antibodies (16). In CDCXM, isolated donor lymphocytes are separated into T and B cells and mixed with recipient serum. Subsequently, complement derived from rabbit serum is added. If donor-specific antibodies bind to donor cells, the complement cascade will be activated via the classical pathway, leading to lymphocyte lysis (Fig. 1). The results are reported as the percentage of dead cells to live cells. A score of 2 is taken as the cutoff for a positive result (about 20% of cells undergo lysis), while the score of 8 defines complete cell lysis (Tab. I). The DCDXM is also used for semi-quantitative reaction strength assessment, by titrated crossmatch using serial serum dilutions, which could be beneficial in predicting negative crossmatch following desensitization. The sensitivity of basic CDCXM is not good and depends on the living donor cells and high antibodies titres (17,18). However, the sensitivity of basic CDCXM can be increased by adding antihuman globulin (AHG).

Tab. I. CDCXM scoring system (**Source:** Terasaki et al. 2005).

SCORE	DEAD CELL(%)	DESCRIPTION/INTENSITY OF THE REACTION
1	< 10	Negative
2	10-20	Doubtful Positive
4	20-40	Weakly Positive
6	40-80	Positive
8	80-100	Strongly Positive

Tab. II. Methods To Reduce False Reactivity (**Source:** Authors Personal Collection).

METHOD	MECHANISM	COMMENTS
Heat Inactivation	Aggregation of IgM	Aggregates formed may bind non-specifically
Chemical Inactivation (Dithiothreitol and Dithioerythritol)	Reduction of disulfide bonds IgM	carcinogenic agents and may cause the loss of some IgG
Hypotonic Dialysis	Filtration of IgM	Small amounts of IgM left in filtrates
Amos (3-Wash) and Amos- modified (1-Wash)	Elimination of anticomplementary factors	Decreases false negative CDCXM
Prolonged incubation technique	Promotion of complement fixation	Decreases false negative CDCXM

T-cell CDC: T lymphocytes express only class I HLA molecules,

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and a positive T-cell crossmatch incurs a very poor outcome. Patel et al. studied outcomes in 30 transplant recipients with a positive T-cell crossmatch, and they reported immediate graft loss in 24 cases and early graft rejection, within 3 months, in the remaining patients. False positive reactions or lower immunogenicity of antigens or antibodies could cause delayed rejection (19).

B-cell CDC: B lymphocytes express both class I and class II HLA molecules. B-cell crossmatching detects antibodies against class II HLA molecules. Positive results are not as decisive as in the case of T-cell crossmatching due to a higher rate of false positive results (50%) and time constraints in the case of deceased organ transplantation (20). Negative results are reassuring, i.e., even if the T-cell crossmatch is positive, this will be due to non-HLA antibodies alone (21,22). Most centres perform B-cell crossmatching in living donor transplant assessment. In the case of positive results, the presence of DSA is better determined by more specific means such as the Luminex and flow crossmatch assays (23). However, B-cell crossmatching has many limitations as it detects only complement-activating isotypes of antibodies, requires a high degree of vital donor cells, and may show false positive results due to autoantibodies present in patients with autoimmune diseases (24,25,26).In the United Network of Sharing (UNOS) registry, 55% of CDCXM-positive transplant cases were FCXM--negative (27). The present case, with a positive CDCXM and a negative FCXM, could be explained by a false positive CDCXM, a false negative FCXM, or by IgM as the responsible antibody. To confirm autoantibodies as the cause of false positive results, an auto-crossmatch was needed (28) (.

FLOW CYTOMETRY CROSSMATCH TECHNIQUE (FCXM)

In 1983, Garovoy et al. showed that the flow crossmatching technique facilitates transplantation (29). In FCXM, donor lymphocytes and recipient serum are mixed in order to facilitate antibody binding. Subsequently, fluorescein-tagged anti-human globulin (AHG) is added to bind the attached DSA, which are detected by flow cytometry. Detection of fluorescently labelled detection antibodies can identify isotype-specific antibodies. Further subtyping of IgG can also be done.

This further predicts the likelihood of complement activation as IgG4 antibodies do not activate complement in vivo (Fig. 2). Positive FCXM results are important when the CDCXM is negative, i.e., positive results are likely due to a non-complement fixing antibody, a non-HLA antibody, or a low-level antibody. In non-sensitized individuals, positive results are of no significance, while in sensitized individuals, they do suggest inferior graft survival. This can be explained by a higher rate of false positivity in non-sensitized individuals. Sometimes, the CDCXM is positive and a standard FCXM is negative, as in the present case scenario, which could be explained by IgM antibodies that are usually not detected on standard FCXM under anti-IgG tool because IgM antibodies are not of pathological significance in transplant science (30).

To date, there are no clear recommendations regarding a routine use of this technique but some centres use it quite often in the context of donor-specific antibody results and CDCXM in order to predict an overall likelihood of immune complications.

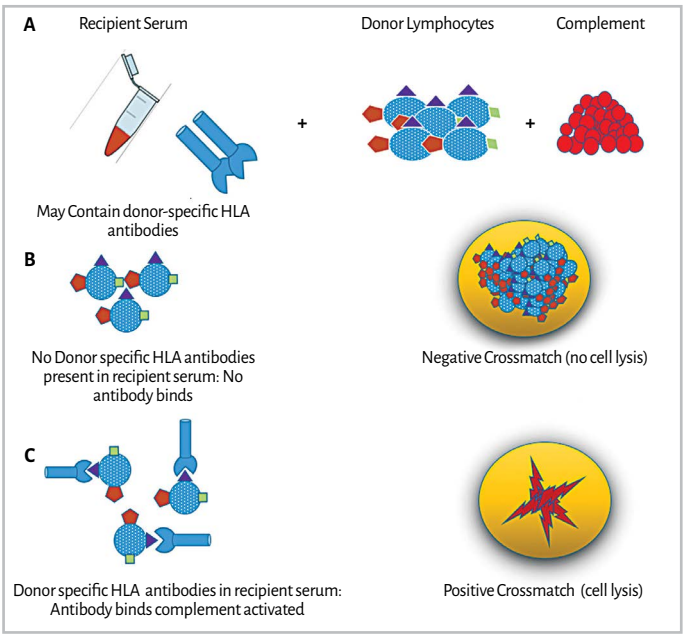

Fig. 1. Steps of CDC Crossmatch (**Source:** Authors own collection).

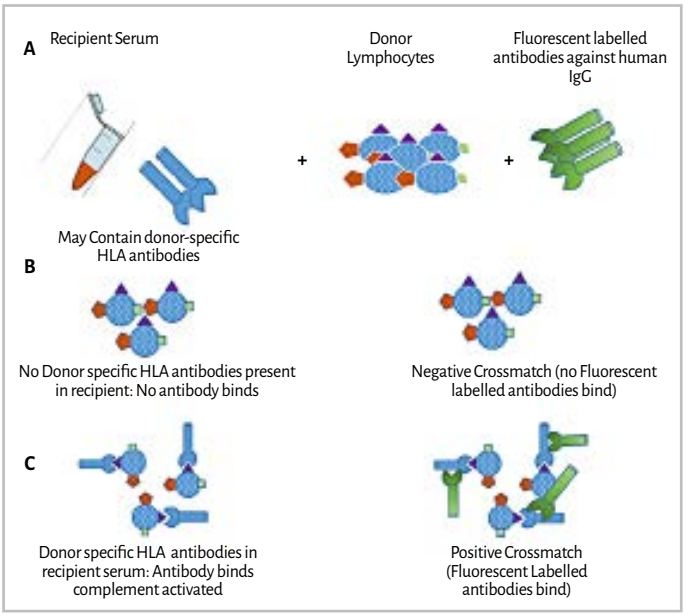

Fig. 2. Steps of the FCXM (**Source:** Author's own collection).

SOLID PHASE ASSAY

Highly sensitive techniques such as enzyme-linked immunosorbent assay (ELISA) and Luminex were developed in order to address the limitation of the CDC procedure. Many laboratories have implemented these methods in their protocol for all tissue typing (7).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

In ELISA, purified HLA class I or class II molecules are immobilized on the surface of microparticles/beads. ELISA is highly sensitive, and allows for antibody identification in 20%-30% of cases of AHG-augmented CDC-negative antibody screening. There are many ELISA-based flow cytometry methods, such as FlowPRA or LAT-M, but they are technically complex and expensive (31).

LUMINEX- SAB (SINGLE ANTIGEN BEAD)

Luminex technique uses antigen coated beads (microspheres) with either multiple HLA antigens for screening purposes or a single HLA antigen in order to increase specificity (32). This technique has been used in many ways as in determining the specific anti--HLA antibodies or virtual crossmatching. These coated beads with unique fluorochrome are mixed with recipient serum. When anti-HLA antibodies present in serum bind to the beads and are detected by an isotype- specific (e.g. IgG) detection antibody via flow cytometry they fluoresce. (Fig. 3) Antibodies are defined against HLA class I and II antigens (33). This virtual crossmatch is used as the reference for comparison of the anti- HLA antibodies of the recipient, with the HLA of the donor. Amajor advantage of the Luminex-SAB is that, in the case of negative results, one could omit CDCXM testing, which reduces cold ischemia time and the need of immunosuppression. False positive results are among the major limitation of the Luminex-SAB; they are due to technical reasons as they do not detect all HLA-directed antibodies but only those against the most common HLA molecules (34,35,36).

The results are reported as molecules of equivalent soluble fluorescence (MESF) and can be graded into weak, moderate, or strong, which helps in antibody titre correlation. The most commonly used MESF cutoff is 1,000 although studies have shown that MESF values well above this level can be associated with a negative CDCXM. Even if there is no reaction on crossmatching, DSA presence on Luminex may have a prognostic significance for the transplanted kidney (37). Studies have reported that recipients with donor-specific anti-HLA antibodies have worse graft survival compared to recipients with non-donor specific anti-HLA antibodies, which is still worse than no DSA (38,39). The main advantage of the Luminex technique is its ability to detect specific antibodies, which eliminates the risk of false positivity. However, the Luminex technique has some limitations as well; for instance, incomplete or varied antigen representation on beads or presence of IgM antibodies can affect results (40,41). False negative results are also reported in the case of high HLA antibody titres due to the prozone effect or IgM antibodies, which hinders anti-HLA antibody binding to beads, or due to epitope sharing between different antigen beads (42).

PANEL REACTIVE ANTIBODIES (PRA)

In an analysis of panel reactive antibodies (PRA), recipient serum is mixed with a panel of lymphocytes representing a potential donor HLA makeup. The result is reported as the percentage of PRA reactions (%PRA). In non-sensitized candidates, it is 0, and

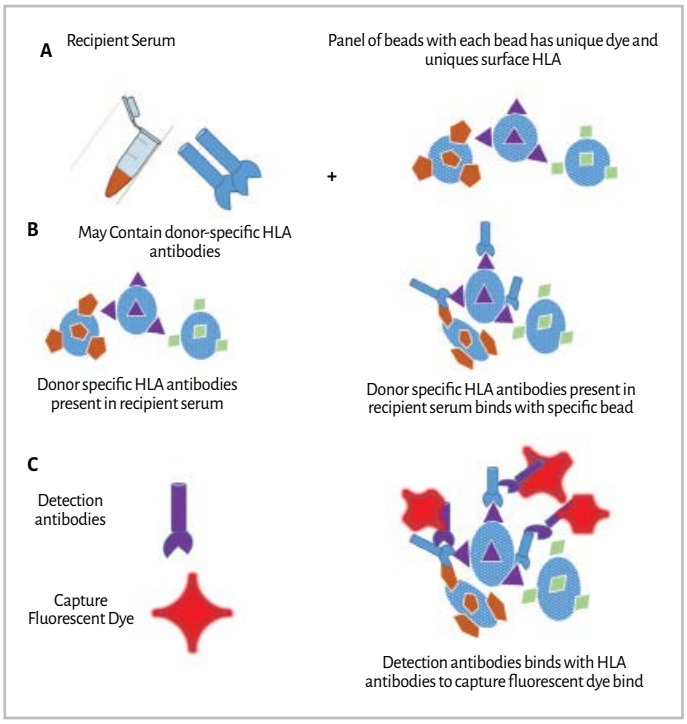

Fig. 3. Steps of Virtual Crossmatch (**Source:** Authors own collection).

if candidate's serum reacts in 80 out of 100 cases, it is 80%. Clinically, this result is interpreted as a high likelihood of rejection, i.e., 8 out of 10 times. Technological advancement has improved the determination of antibody specificity. Maintaining records of antigens increases the efficiency of organ allocation by screening off incompatible donors; otherwise, recipients would be at a high risk of hyperacute rejection. The frequency of unacceptable antigens in the national donor pool can be used for determination of calculated panel reactive antibodies (CPRA), i.e., the likelihood of incompatibility, by using a computer-based algorithm (43,44).

CONCLUSION

We evaluated the effect of DSA positivity in the case of a negative CDCXM. However, there are few report regarding the impact of DSA negativity in the case of a positive CDCMX. In 2011, Amico et al. reported an excellent graft outcome with a negative virtual crossmatch (39). Few anecdotal reports exist in which a desensitization protocol was used for such cases. Furthermore, even post--desensitization, there was no change in tissue matching status; thus, it was not needed. However, either omission of a positive CDC crossmatch or choosing desensitization is the clinician's discretion.

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