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Solid Phase-Based Cross-Matching Using the Luminex Platform for Solid Organ Allo-Grafting: Rather Regression than Progress in Comparison to the ELISA-Based Precursor Procedure

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Abstract

Antibodies directed against HLA antigens of a given donor represent the most prominent cause for hyper-acute and acute rejections. In order to select recipients without donor-specific antibodies the complement-dependent cytotoxicity (CDC-) crossmatch was first established representing the standard procedure up to the present. Its negative pre-transplant outcome is currently regarded as the most important requirement for a successful short term kidney graft survival. As a functional assay, however, it strongly depends on the availability of isolated donor lymphocytes and in particular on their vitality. Moreover, during the last ten years several disadvantages of the CDC-based procedure have increasingly been discussed with respect to this assay's high susceptibility to disruptive factors which frequently lead to false positive outcomes. In this context several autoimmune diseases especially of the immune complex type (type III) or pharmacological treatment of a given recipient have been shown to lead to unexpected "false-positive" outcomes of the CDC-crossmatch. As methodical alternatives for anti-HLA antibody specific cross-matching two ELISA-based procedures i) the Antibody Monitoring System (AMS-) ELISA and ii) the AbCross-ELISA were established in our tissue typing laboratory and those of some other groups. Both systems, however, were discontinued for mere commercial reasons in the years 2013 and 2016, respectively. Using the same set of diagnostic antibodies, the AMS-ELISA, now named Donor-Specific Antibodies/DSA, was afterwards again manufactured as a microbead-based array using the Luminex platform. With a view to establish the DSA-assay as the only remaining solid phase-based crossmatch system commercially available, this procedure was systematically evaluated in our laboratory. Primarily but not exclusively based on drawbacks of the evaluation software, however, 69 (32.5%) of the virtually defined crossmatch results (n=212 independent anti-HLA class I and II specifications and their corresponding DSA-assays, respectively) were classified as divergent using the DSA-assay whereas only 143 results (67.5%) were classified as accordant by this assay's software. Referring to the chosen cohort of recipients (n=106) not less than 62 (58.4%) of them are characterized by findings which are not supported by virtual cross-matching. We here provide evidence that for various reasons the outcomes provided by the DSA-assay, in contrast to those of the AMS-ELISA as its precursor system, have critically to be challenged. We therefore conclude that modifications are urgently required to be introduced by the manufacturer in order to lead again to a system of sufficient validity usable for any laboratory's routine diagnostics.

Keywords: Allo-grafting; Complement-dependent cytotoxicity assay; Cross-matching; Donor-specific antibodies; ELISA; Human leukocyte antigens; Major histocompatibility complex; Rejection; Solid phase-based cross-matchingAbbreviations: AMS: Antibody Monitoring System; CDC-XM: Complement-Dependent Cytotoxicity Crossmatch; DSA: Donor-Specific Antibodies; HLA: Human Leukocyte Antigens; MHC: Major Histocompatibility Complex; SPA: Solid Phase Assays; PBL: Peripheral Blood Lymphocytes; XM: Crossmatch

Introduction

It has been known for about fifty years that antibodies directed against Human Leukocyte Antigens (HLA) are predominantly responsible for hyper-acute or acute rejections of renal allografts and allografts of other organs [1-3]. In accordance with the transplantation guidelines of most countries and supranational societies (e.g. Eurotransplant Foundation) which supervise the allocation of allografted organs Donor-specific anti-HLA antibodies (DSA) are therefore generally regarded as clear contraindication. In order to detect DSA in a chosen recipient's serum against lymphocytes from a given donor's blood the so-called crossmatch (XM-) procedure was developed already in the late 1960s [1]. The technique first established in those days which later became the methodical standard was the complement-dependent cytotoxicity crossmatch (CDC-XM). As detected by their functional characteristics only those DSA are demonstrable which exert their detrimental function through the activation of the complement system's classical pathway. Thus, only so-called cytotoxic antibodies are generally traced by lysed (dead) target donor lymphocytes which are finally identified by two color fluorescence microscopy. This technique, however, fails to detect DSA which do not exhibit complement-fixing activity although these may just as well be detrimental to tissues or organs of donors. Additionally the CDC-CM is characterized by its rather low sensitivity leading to the inability to detect low concentrations of DSA. This early known drawback resulted in this assay's modification using secondary anti-human immunoglobulin antibodies which are directed against the primary DSA, a design, which was termed anti-human globulin (AHG)-enhanced CDC-XM [4,5]. The additional incubation time,

however, resulting from this modification was especially disadvantageous if the quality of the cells under investigation was poor right from the beginning. Accordingly the background of dead cells rose to a level which at least partly covered the antibody-mediated complement reactions highly restricting the detectability of faint antibodies. As a matter of fact insufficient cell qualities due to inadequate or extended storing of the donors' blood or spleen samples used for their lymphocytes' preparations have been a serious problem of this procedure all over the years. This general drawback holds true till this day although new techniques such as the RosetteSep technique (Stemcell Technologies, Cologne, Germany) have been established in order to isolate PBL, T-cells and B-cells in a less invasive way thereby reducing the portion of dead cells.

As an alternative approach to circumvent CDC-XM-specific problems the flow cytometric crossmatch (FACS-XM) was established. This assay allowed both the detection of complement-independent and of complement-activating i.e. cytotoxic DSA [6-8]. A general drawback of this assay, the sensitivity of which is in the range of the AHG-enhanced CDC-based crossmatch, however, is its artefact-influenced outcome through the "irrelevant/unspecific" binding of antibodies through their Fc-parts to the Fc-receptors expressed on B-lymphocytes which all over the years represented rather a common than a rare event [9,10]. The proposal to perform FACS-based B-cell cross-matching only after pre-incubation of these donor cells with heat-denatured rabbit serum has been the first reliable approach to overcome this methodical drawback [11]. This procedure, well known to block Fcγ-receptors for immunohistochemical applications first had the capacity to overcome the problem of the unspecific binding of antibodies through their Fc-parts in contrast to the procedure using *pronase* as proposed formerly [12,13]. As *pronase* is a commercially available mixture of proteases isolated from the extracellular fluid of *Streptomyces griseus* its activity extends to both denatured and native proteins leading to nearly complete enzymatic digestion into individual amino acids. Thus, the reproducibility of improving FACS-based crossmatch outcomes by pre-treating B-cells with *pronase* was difficult due to the loss of Fc-receptors as well as of HLA molecules. A standardized protocol has therefore never been developed for this procedure suggesting that it generally has never provided the capacity to represent any standard procedure in order to improve the outcomes of flow cytometry cross-matching.

A further drawback of the FACS-XM, which holds especially true for the CDC-XM, is the dependence of both assays on the availability of vital donor cells. If only cells of poor quality (i.e. with a vitality rate lower than 80%) or exclusively acellular donors' tissues are available both assays do not lead to valid results. Due to this methodical aspect i.e. in order to act independently of the availability of separated vital cells or their insufficient quality, additional crossmatch assays were established using the design of solid phase-based assays (SPA). Two diagnostic systems, both representing enzyme-linked immunosorbent assays (ELISA) were implemented in our tissue typing laboratory for various groups of patients suffering from artificially influenced CDC-crossmatch outcomes [9,14-18] or characterized by the general lack of single donors' lymphocytes [19]. Both assays, however, the pros and cons of which will afterwards be discussed, were discontinued by their manufacturers for mere commercial reasons in the years 2013 and 2016, respectively. Thus, the only remaining diagnostic solid phase-based system afterwards available has been the Luminex microbead-based array named DSA (Donor Specific Antibodies) which was evaluated by our laboratory in order to implement it as a technical alternative to CDC-cross-matching for special patients' cases as mentioned above after the discontinuation of both ELISA-based systems. We here present the results of our evaluation cohort of test recipients chosen by virtual cross-matching. Apart from only few recipients nearly all of them were characterized by single antigen specificities against only one phenotype of the various gene loci of only one class of HLA-molecules (A, B, C for HLA-class I and DR, DQ for HLA- class II, respectively).

Materials and Methods

Patients' sera and spleen cells of post-mortem organ donors

Deep-frozen (-28°C) stored sera of 106 patients were chosen as recipients' sera with known antibody specificities (Table 1) defined at previous quarterly antibody screening runs. These antibody specifications are obligatory for patients of the Eurotransplant kidney waiting list following the guidelines of the German Federal Medical Association and those of Eurotransplant. It is noteworthy that these arrangements comprising 106 patients led to 212 independent combinations/runs for both HLA-classes, respectively.

Donor 1			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A24,26; B27,38; Cw2,12	DR1,4; DQ5,7	HLA-class I/ class II
Recipients' antibody specificities			
K.R.	anti-A24		+/Ø
S.S.	anti-A26		+/Ø
F.R.	anti-A24		+/Ø
P.M.	anti-A24		+/Ø
M.F.	anti-B38		+/Ø
L.I.	anti-B27,28		+/Ø
W.R.		anti-DR4	Ø/+
N.S.		anti-DR1	Ø/+
M.B.		anti-DR4	Ø/+
P.M.		anti-DR4	Ø/+
B.M.		anti-DQ5	Ø/+
H.T.		anti-DQ7	Ø/+
K.R.		anti-DQ7	Ø/+
L.I.		anti-DQ5	Ø/+
S.E.		anti-DQ5	Ø/+
S.S.	anti-A24, -B27	anti-DQ5	+/+
S.I.	anti-Cw2,12	anti-DR1,4; -DQ5,7	+/+
Donor 2			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results

	A2,24; B13,17; Cw2,6	DR4,7; DQ2,8	HLA-class I/ class II
Recipients' antibody specificities			
L.S.	anti-A2,24		+/Ø
N.S.	anti-A2,24		+/Ø
S.S.	anti-A24		+/Ø
R.G.	anti-A2		+/Ø
D.H.	anti-A2		+/Ø
B.R.	anti-A2,24		+/Ø
E.G.	anti-A2,24		+/Ø
S.S.	anti-B13		+/Ø
S.B.	anti-B13		+/Ø
S.B.	anti-B13,27		+/Ø
K.S.	anti-B13		+/Ø
K.J.		anti-DR4	Ø/+
M.C.		anti-DQ2	Ø/+
M.I.		anti-DQ2	Ø/+
R.K.		anti-DQ2	Ø/+
J.U.	anti-B13,27	anti-DR4	+/+
S.M.	anti-Cw2,6	anti-DR4	+/+
Donor 3			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A2; B7,62; Cw3,7	DR13,15; DQ6	HLA-class I/ class II
Recipients' antibody specificities			
E.G.	anti-A2		+/Ø
B.M.	anti-B7		+/Ø
S.A.		anti-DR13	Ø/+
S.E.		anti-DQ6	Ø/+
L.M.		anti-DQ6	Ø/+
Donor 4			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A2,29; B44; Cw5,6	DR,7; DQ2,8	HLA-class I/ class II
Recipients' antibody specificities			
R.P.	anti-A2		+/Ø
P.M.	anti-B44		+/Ø
S.B.		anti-DR7	Ø/+
G.T.		anti-DR4,7	Ø/+
K.T.		anti-DQ2,8	Ø/+
B.M.		anti-DQ8	Ø/+
Donor 5			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A2,11; B35,60; Cw3,4	DR1,13; DQ5,6	HLA-class I/ class II
Recipients' antibody specificities			
K.J.	anti-A11		+/Ø
K.D.	anti-B60		+/Ø
B.N.		anti-DR1,13	Ø/+
K.B.		anti-DR1	Ø/+
M.I.		anti-DQ5,6	Ø/+
K.S.		anti-DQ5,6	Ø/+
Donor 6			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A2,30; B37,51; Cw5,6	DR10,13; DQ5,6	HLA-class I/ class II
Recipients' antibody specificities			
K.S.	anti-B51		+/Ø
P.E.		anti-DR13	Ø/+
W.R.		anti-DR10	Ø/+
D.A.		anti-DQ6	Ø/+
B.S.		anti-DQ6	Ø/+
R.R.	anti-Cw6	anti-DR13	+/+
Donor 7			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A1,32; B8,44; Cw5,7	DR11,17; DQ2,7	HLA-class I/ class II
Recipients' antibody specificities			
K.R.	anti-A32		+/Ø
H.J.	anti-A1		+/Ø
F.R.	anti-A1		+/Ø
S.B.	anti-B8		+/Ø
S.M.	anti-B8		+/Ø
S.K.	anti-B8		+/Ø
S.A.		anti-DR11,17	Ø/+
L.S.		anti-DR11,17	Ø/+

B.M.		anti-DR11	Ø/+
B.N.		anti-DR11,17	Ø/+
N.M.		anti-DR17	Ø/+
K.B.		anti-DR11	Ø/+
K.J.		anti-DQ7	Ø/+
K.D.		anti-DQ7	Ø/+
K.T.		anti-DQ2,7	Ø/+
K.S.		anti-DQ2	Ø/+
W.H.		anti-DQ2,7	Ø/+
R.K.		anti-DQ2	Ø/+
G.H.	anti-A32	anti-DR11,17	+/+
N.M.	anti-B44	anti-DR11	+/+
F.R.	anti-A1,32	anti-DR17	+/+
Donor 8			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A3,24; B35,60; Cw3,4	DR1,15; DQ5,6	HLA-class I/ class II
Recipients' antibody specificities			
K.J.	anti-A3		+/Ø
E.G.	anti-A24		+/Ø
M.B.	anti-A3		+/Ø
G.T.	anti-A24		+/Ø
S.S.	anti-A24		+/Ø
S.M.	anti-A3		+/Ø
B.M.	anti-B60		+/Ø
M.C.	anti-B35		+/Ø
S.B.	anti-B60		+/Ø
S.J.	anti-B35		+/Ø
T.S.	anti-B35,60		+/Ø
B.K.	anti-B35		+/Ø
K.S.	anti-B35		
D.H.		anti-DR15	Ø/+
B.N.		anti-DR1,15	Ø/+
K.B.		anti-DR1	Ø/+
K.R.		anti-DQ6	Ø/+
E.A.		anti-DQ6	Ø/+
B.S.		anti-DQ6	Ø/+
K.S.		anti-DQ5,6	Ø/+
M.I.		anti-DQ5,6	Ø/+
K.D.	anti-B60	anti-DR15	+/+
Donor 9			
HLA-phenotypes	HLA-class I	HLA-class II	Virtual XM-results
	A28,32; B44,64; Cw4,8	DR11,13; DQ7	HLA-class I/ class II
Recipients' antibody specificities			
L.R.	anti-A32		+/Ø
K.R.	anti-A28		+/Ø
B.N.		anti-DR11,13	Ø/+
K.B.		anti-DR11	Ø/+
S.R.		anti-DQ7	Ø/+
W.H.		anti-DQ7	Ø/+

Note: XM=crossmatch; +=positive result; Ø=negative result

Table 1: HLA class I and II antigens of nine donors and antibody specificities of their chosen virtually positive recipients (n=106) in order to create the corresponding virtually defined crossmatch results.

Donors' splenic leukocytes were taken from residual tissue of retained samples of 9 selected *post mortem* organ donors appearing during emergency duties. They all were stored as cell pellets (-28°C) until used for *de facto* cross-matching with the Luminex-based DSA assay. For this assay both the sera of 106 recipients and the cell pellets of the 9 *post mortem* donors were virtually arranged in a way that nearly all of the recipient/donor combinations exhibited donor-specific antibodies only against one or two HLA-antigens of one single gene locus of only one class of HLA-antigens, respectively. Thus, only 9/106 (8.5%) of the recipients showed virtually definable DSA against antigens of both HLA classes I and II, and various DSA directed against combinations of antigens comprising different gene loci (A, B and C for HLA-class I and DR, DQ for HLA-class II) were a priori highly reduced by so-called virtual cross-matching leading to the defined DSA shown in Table 1.

HLA-typing of the post-mortem organ donors and of the recipients

All recipients and post-mortem donors were pheno- and genotyped for HLA class I antigens comprising the loci A, B and C. Phenotyping was performed using Histo Tray ABC 144 plates (BAG, Lich, Germany). Genotyping was performed using sequence-specific primer- (SSP-) PCR-based low resolution technique (Innotrain, Kronberg, Germany and Protrans, Ketsch, Germany) both for the HLA class I loci A, B and C and for the HLA class II loci DRB1* (DR), DRB3* (DR52), DRB4* (DR53), DRB5* (DR51) and DQB1* (DQ) resulting in complete low resolution typing results for the 9 post mortem donors (Table 1) and 106 patients who had been selected as recipients for the evaluation of the DSA assay. The typing results of the recipients only collected for plausibility reasons are not shown as they were not relevant in the context of these investigations.

Detection/Specification of anti-HLA antibodies in order to perform virtual cross-matching

All the recipients' sera were generally screened for anti-HLA class I antibodies using the Quikscreen ELISA, and for anti-HLA class II antibodies using the B-screen ELISA (both from Biorad, Dreieich, Germany). In a secondary step patients' sera were investigated in order to specify these antibodies. This was done using the Luminex-based anti-HLA specification system *Luminex-ID* (Immucor, Rödermark, Germany) at the single donor (ID) resolution using microbeads on which native i.e. HLA class I or class II antigens isolated of one single donor had been immobilized, respectively. We used this ID-system instead of the Single Antigen (SA) system to avoid the pseudo-specification of so-called natural antibodies. These increasingly discussed antibodies are directed against cryptic epitopes of recombinantly generated HLA-antigens but not against corresponding native epitopes of HLA-class I antigens isolated from thrombocytes or HLA-class II antigens isolated from lymphoma cells as these do not exist [20-23]. Thus, right from the beginning false-positive virtual crossmatch outcomes due to the detection of these artificial natural antibodies not representing any HLA-mediated allo-immunization of the recipients were strictly avoided by solely using the Luminex single donor (ID) specification system. It is noteworthy that the limited number of antigen specificities (low panel reactivity/PRA-value) of all recipients' sera used (generally no more than one or two HLA-antigens, three antigens in five cases up to four antigens detectable in only one exceptional case) did not require the highest level of resolution which is only available through the use of the Luminex Single Antigen system.

De facto cross-matching using the Luminex-based Donor-Specific Antibodies (DSA-) detection system

Solid phase-based cross-matching using the Luminex-based DSA system was done according to the manufacturer's instructions by introducing a few modifications. Nearly all steps were performed using 1.5 ml reaction tubes. Initially the Dried Lymphocyte Control (DLC) sample as "donor" material delivered by the manufacturer was prepared by adding 500 µl of cell culture medium and allowed to rehydrate for one hour at room temperature (RT) with occasional vortexing until a uniform suspension of rehydrated cells was reached. The cell mixture was centrifuged at 2,500 rcf for 5 min to pelletize the cells, the supernatant was discarded. The resulting cell pellet of an approximate volume of 50 µl was consecutively used by adding 500 µl of diluted Lymphocyte Lysis Buffer (LLB) [50 µl concentrate with 450 µl reagent grade water] and allowed to completely lyse the cells for 30-45 min at RT. The lysed suspension was maximally centrifuged at 16,000 rcf for 10 min to sediment the cell membranes. The supernatant required as positive control material was frozen at -30°C in adequate aliquots of 50 µl for single use. Prior to use also these aliquots had to be maximally centrifuged (16,000 rcf) for 10 min in order to sediment unwanted aggregates.

For the current investigations donor leukocytes were exclusively isolated from residual splenic tissue instead of gaining donor lymphocytes from peripheral blood in order to avoid the lack of HLA class II antigen-bearing antigen presenting cells (i.e. essentially B-cells). Thus, leukocytes were isolated without the resulting waste of cells through the use of lymphocyte density separation media. Instead leukocytes were isolated by mincing splenic tissue in Red Cell Lysis Buffer/RCLB (10 × RCLB=50 ml 2M TRIS/pH 7.5; 33 ml 3 M NaCl; 10 ml 5 M MgCl; ad 1000 ml H_20) followed by a washing step using RCLB in 50 ml tubes. In order to prepare the lysates of the spleen-derived leukocytes all the donors' cell pellets were transferred into a 1.5 ml reaction tube and deep-frozen (-30°C) until usage. Cell lysates were prepared by dislocating the cell pellet with 10-fold its volume using Lymphocyte Lysis Buffer (10-fold diluted). The resulting suspension was incubated for 20 min with occasional vortex-motion agitations followed by a centrifugation step at 16,000 rcf for 10 min to sediment the unwanted cell membranes.

Binding of the lysed HLA-antigens to the Luminex-beads was performed in 1.5 ml tubes by calculating 8 µl of donor's lysate and 5 µl of bead suspension per control antibody and recipients' sera under investigation. An exemplary incubation scheme comprising all controls for one donor lysate against which 6 sera are tested is shown in Figure 1. In order to compensate the loss of volumes due to the consecutive pipetting steps at least one additional volume of donor's cell lysate and bead suspension should be prepared in addition. The mixtures of donors' lysates and bead suspensions were incubated on a shaker for 30 min in the dark with occasional vortex motions. During this incubation the additional positive control termed Lysate Control Reagent (LCR), a biotinylated monoclonal antibody directed against HLA-class I or class II molecules, respectively, was prepared at a 1:10 dilution using Specimen Diluent (SD) provided by the manufacturer. These control antibodies indicate that a sufficient amount of HLA-molecules were immobilized by the capture antibodies. Thus, a loss of the LCR-based control signals from the outset points to the impracticability to receive a signal based on DSA by the recipients' sera. A scheme of the workflow comprising both the use of recipients' sera and the LCR-controls is shown in Figure 2. After 30 min of incubation the mixture of capture beads/donor lysates were complemented by washing buffer (42 µl per calculated well of a microtiter plate provided by the manufacturer) in order to reach a final volume of 55 µl which was portioned into each individual well (Figure 1). After the transfer the beads with the immobilized HLA-antigens were washed three times by filling the wells with 150 µl washing buffer and consecutives centrifugation steps at 1,100 rcf for 5 min each. Supernatants were jerkily poured out strictly avoiding any loss of beads. In the meantime prepared 1:3 and 1:6 dilutions of the recipients' serum samples as well as the LCR-dilutions (all in Specimen Diluent) were filled into the respective wells as exemplarily shown in Figure 1 at a final volume of 50 µl. Additionally, positive (PC) and negative control sera (NC) were prepared (12 µl of the control sera and 38 µl SD each) and used for the DLC control lysate as well as for each of the donors' lysates under investigation (Figure 1). As shown above the following incubation was performed again for 30 min on a shaker in the dark with occasional vortex motions to avoid any aggregation of the beads. Afterwards three consecutive washing steps were carried out as mentioned above in order to remove unbound antibodies. In the meantime the secondary reagents labelled with fluorescence dyes had to be prepared. These were i) the Phycoerythrin-conjugated goat anti-human IgG secondary antibody (CJS) required to detect bound recipients' donor-specific anti-HLA antibodies and ii) Phycoerythrin-conjugated Streptavidin (SA-PE) essential to detect bound monoclonal biotinylated control antibodies (Lysate Control Reagent/LCR). SA-PE was diluted 1:10 using washing buffer and had to be prepared for a calculated volume of 50 µl per well (i.e. 100 µl according to Figure 1, red stars). Again using washing buffer CJS was prepared at the same dilution leading to a total volume of 800 µl according to the scheme of Figure 1 (wells without red stars). Again an incubation (30 min on a shaker in the dark with occasional vortex motions) followed in order to

bind the fluorescence dye-labelled secondary reagents (secondary antibodies or streptavidin, respectively) to the primary antibodies (DSA) out of the recipients' sera or the monoclonal control antibodies (Figure 2), and again this last incubation step was followed three washing steps as shown above. In order to circumvent any aggregation especially prior to the measurement the bead suspensions of all wells were vigorously resuspended using an 8-gauge pipette.

	1	2	3	4	5..
A	DLC-Lysate PC	Donor's Lysate Serum 2 (1:3)	Donor's Lysate Serum 6 (1:3)		
B	DLC-Lysate NC	Donor's Lysate Serum 2 (1:6)	Donor's Lysate Serum 6 (1:6)		
C	DLC-Lysate LCR ★	Donor's Lysate Serum 3 (1:3)			
D	Donor's Lysate PC	Donor's Lysate Serum 3 (1:6)			
E	Donor's Lysate NC	Donor's Lysate Serum 4 (1:3)			
F	Donor's Lysate LCR ★	Donor's Lysate Serum 4 (1:6)			
G	Donor's Lysate Serum 1 (1:3)	Donor's Lysate Serum 5 (1:3)			
H	Donor's Lysate Serum 1 (1:6)	Donor's Lysate Serum 5 (1:6)			

Figure 1: Exemplary incubation scheme comprising all controls and 6 sera of chosen recipients at two dilution steps (1:3 and 1:6) for one given donor. DLC (Dried Lymphocyte Control): freeze-dried control lymphocyte pellet delivered by the manufacturer which has initially to be prepared; LCR (Lysate Control Reagent): Biotinylated monoclonal antibody directed against HLA-class I or II antigens, respectively; NC: negative control serum; PC: positive control serum; Red Stars: last incubation step using SA-PE (phycoerythrin-conjugated streptavidin) in order to detect LCR (biotinylated monoclonal control antibodies).

Calculating the data and classifying them as "negative" or "positive" for DSA was done by an algorithm of the corresponding software of the DSA assay. These classifications were based on the individual recipients' raw data with simultaneous consideration of a so-called "background determining factor" leading to individual cut off values for each recipient. Furthermore, additional beads which are not coated with capture antibodies are included in the DSA assay. These control beads provide values which contribute to the recipients' classification as "negative" or "positive" for DSA. The respective beads termed control beads were:

coated with human albumin (Con1).

coated with the thrombocyte-derived antigen glycoprotein IV (Con2).

naked beads not covered by any protein (Con3).

Additional beads (position 77) were coated with human IgG in order to test the correct application and binding of the secondary antibody [Phycoerythrin-conjugated goat anti-human IgG secondary antibody (CJS)]. Other beads (position 78) prepared with a biotinylated protein of unknown origin were integrated in order to check the correct application of the detection reagent [Phycoerythrin-conjugated Streptavidin (SA-PE)] used for the LCR controls (Figure 1, red stars). Taken together a widespread network of controls was implemented in order to detect any component of the kit lacking its function.

Results

We systematically evaluated the DSA-system for the detection of donor-specific anti-HLA antibodies in order to compensate two formerly established ELISIGUREA-based systems both of which had been discontinued only for commercial reasons by their respective manufacturers. Thus, as defined by virtual cross-matching combinations of donors and their respective recipients were investigated which are completely listed in Table 1. Spleen cell-derived leukocyte lysates of nine donors were chosen in a way to present only one virtual HLA-target (n=75/70.8%), two targets (n=25/23.6%), three targets (n=5/4.7%) or four targets (n=1/0.9%) for the sera of patients chosen as possible recipients (n=106) (Table 1). Of all recipients only nine (8.5%) virtually exhibited antibodies against both classes of HLA-antigens. These combinations were chosen with the intent to preferentially investigate mono- or bispecific sera thus avoiding unforeseeable effects which may arise from well-known errors of antibody specifications of highly immunized recipients. It was our initial and clear aim to trace back positive results of *de facto* i.e. DSA-based cross-matching to a single or a highly limited number of antibody specificities detectable by virtual cross-matching or to exclude these defined antigen(s). Especially the virtual exclusion of DSA could reliably be done only in cases with limited i.e. clearly definable antibody specificities of a given recipient. Furthermore, the antibody specification procedure chosen (ID/PRA-level) by using immobilized antigens of single donors (HLA class I or II antigens, respectively) instead of recombinantly prepared single antigens led to reliable results only in cases with limited specificities due to this specification assay's limited capability of resolution. However, in order to strictly exclude errors based on so-called natural antibodies and subsequent virtual crossmatch results of artificial positivity this level of resolution was chosen with intent. The relative intensities of the signals defined by their MFI-Values were for all virtually defined specificities higher than 4,000 and thus represented signals far above any probable background-derived values. Additionally, the recipients' virtually defined specificities had to be detectable for at least three following quarters with this intensity (MFI=4,000) in order:

To guarantee the availability of sufficient volumes of the recipients' pooled serum samples required for all analyses.

To exclude an artefact-based unique identification of anti-HLA antigen specificities.

Taken together the virtually determined DSA or their exclusions (Table 1), respectively, were to our best knowledge substantial and not based on various possible sources of artefacts. Thus, their virtual identification should almost certainly be substantiated by their DSA-based *de facto* detection.

Comparative analyses of both procedures, however, led to unexpected results. As the detection of DSA against HLA-class I or Class II antigens was performed by completely independent assays (i.e. using different sets of capture as well as of control antibodies) 106 recipients led to 212 independent results as is shown in Table 2 where the data of virtual and those of DSA-based de facto cross-matching are directly confronted. Of the overall outcome comprising 212 positive and negative results together 143 results (67.5%) are accordant whereas 69 (32.5%) are discrepant. Of the whole number of 69 discrepant results of the DSA-assay 44 (64%) are divergent in a positive way in

contrast to 25 results (36%) exhibiting data which diverge negatively from the virtually positive crossmatch results. Referring to the chosen cohort of recipients (n=106), not less than 62 (58.4%) of them are characterized by findings which are not at all supported by virtual cross-matching (Table 2).

Donor 1	**a) Virtual XM-results**	**b) Results of Immucor DSA-XM**	
Recipients ' ID	**Class I/Class II**	**Class I/Class II**	
K.R.	+/Ø	+/+	
S.S.	+/Ø	+/Ø	
F.R.	+/Ø	+/Ø	
P.M.	+/Ø	+/Ø	
M.F.	+/Ø	+/+	
L.I.	+/Ø	+/+	
W.R.	Ø/+	+/+	
N.S.	Ø/+	Ø/+	
M.B.	Ø/+	Ø/+	
P.M.	Ø/+	+/+	
B.M.	Ø/+	+/+	
H.T.	Ø/+	+/+	
K.R.	Ø/+	Ø/Ø	
L.I.	Ø/+	+/+	
S.E.	Ø/+	Ø/+	Accordant: n=25 discrepant: n=9 (pos.: n=8) (neg.: n=1)
S.S.	+/+	+/+	
S.I.	+/+	+/+	
Donor 2			
L.S.	+/Ø	+/+	
N.S.	+/Ø	Ø/Ø	
S.S.	+/Ø	+/Ø	
R.G.	+/Ø	+/Ø	
D.H.	+/Ø	+/+	
B.R.	+/Ø	+/Ø	
E.G.	+/Ø	+/+	
S.S.	+/Ø	Ø/+	
S.B.	+/Ø	Ø/+	
S.B.	+/Ø	+/Ø	
K.S.	+/Ø	Ø/Ø	accordant: n=24 discrepant: n=10 (pos.: n=5) (neg.: n=5)
K.J.	Ø/+	Ø/Ø	
M.C.	Ø/+	Ø/+	
M.I.	Ø/+	Ø/+	
R.K.	Ø/+	Ø/+	
J.U.	+/+	+/+	
S.M.	+/+	+/+	
Donor 3			
E.G.	+/Ø	+/+	
B.M.	+/Ø	+/Ø	
S.A.	Ø/+	Ø/Ø	Accordant: n=8 discrepant: n=2 (pos.: n=1) (neg.: n=1)
S.E.	Ø/+	Ø/+	
L.M.	Ø/+	Ø/+	
Donor 4			
Recipients ' ID	**Class I/Class II**	**Class I/Class II**	
R.P.	+/Ø	Ø/Ø	
P.M.	+/Ø	Ø/Ø	
S.B.	Ø/+	Ø/+	
G.T.	Ø/+	Ø/+	Accordant: n=8 discrepant: n=4 (pos.: n=0) (neg.: n=4)
K.T.	Ø/+	Ø/Ø	
B.M.	Ø/+	Ø/Ø	
Donor 5			
K.J.	+/Ø	+/Ø	
K.D.	+/Ø	+/Ø	
B.N.	Ø/+	Ø/+	
K.B.	Ø/+	Ø/+	Accordant: n=11 discrepant: n=1 (pos.: n=0) (neg.: n=1)
M.I.	Ø/+	Ø/Ø	
K.S.	Ø/+	Ø/+	
Donor 6			
K.S.	+/Ø	Ø/Ø	
P.E.	Ø/+	Ø/+	
W.R.	Ø/+	+/+	
D.A.	Ø/+	Ø/Ø	Accordant: n=8 discrepant: n=4 (pos.: n=1) (neg.: n=3)
B.S.	Ø/+	Ø/+	
R.R.	+/+	Ø/+	
Donor 7			
K.R.	+/Ø	Ø/Ø	
H.J.	+/Ø	+/Ø	
F.R.	+/Ø	+/+	
S.B.	+/Ø	Ø/+	

S.M.	+/Ø	+/+	
S.K.	+/Ø	+/+	
S.A.	Ø/+	+/+	
L.S.	Ø/+	+/+	
B.M.	Ø/+	+/+	
B.N.	Ø/+	Ø/+	
N.M.	Ø/+	Ø/+	
K.B.	Ø/+	Ø/+	
K.J.	Ø/+	Ø/+	
K.D.	Ø/+	Ø/+	
K.T.	Ø/+	Ø/Ø	
K.S.	Ø/+	Ø/+	
W.H.	Ø/+	Ø/+	
R.K.	Ø/+	Ø/+	
G.H.	+/+	+/+	Accordant: n=32
N.M.	+/+	+/+	discrepant: n=10
F.R.	+/+	+/+	(pos.: n=7)
			(neg.: n=3)
Donor 8			
Recipients ' ID	**Class I/Class II**	**Class I/Class II**	
K.J.	+/Ø	+/+	
E.G.	+/Ø	+/+	
M.B.	+/Ø	Ø/+	
G.T.	+/Ø	+/+	
S.S.	+/Ø	+/Ø	
S.M.	+/Ø	+/+	
B.M.	+/Ø	+/+	
M.C.	+/Ø	+/+	
S.B.	+/Ø	Ø/+	
S.J.	+/Ø	+/+	
T.S.	+/Ø	+/+	
B.K.	+/Ø	+/+	
K.S.	+/Ø	+/+	
D.H.	Ø/+	+/+	
B.N.	Ø/+	+/+	
K.B.	Ø/+	+/+	Accordant: n=21
K.R.	Ø/+	+/+	discrepant: n=23
E.A.	Ø/+	+/Ø	(pos.: n=20)
			(neg.: n=3)
B.S.	Ø/+	+/+	
K.S.	Ø/+	+/+	
M.I.	Ø/+	+/+	
K.D.	+/+	+/+	
Donor 9			
L.R.	+/Ø	Ø/+	
K.R.	+/Ø	Ø/Ø	
B.N.	Ø/+	Ø/Ø	
K.B.	Ø/+	+/+	Accordant: n=6
S.R.	Ø/+	Ø/+	discrepant: n=6
W.H.	Ø/+	Ø/Ø	(pos.: n=2)
			(neg.: n=4)
Note: XM=crossmatch; +=positive result; Ø=negative result; green symbols=in accordance with virtual XM-results; red symbols=discrepant from virtual XM-results			

Table 2: Comparison of the outcomes of a) virtual cross-matching and b) DSA-based
cross-matching using the evaluation software of the manufacturer *Immucor*.

If the antigens of the single gene loci A, B and Cw (HLA-class I) and DR and DQ (HLA-class II) are separately analyzed the number of diverging DSA-based results not detecting virtually positive results was calculated. It is noteworthy that also recipients ' specificities exceptionally comprising more than one gene locus or both HLA-classes are considered by 9 combinations presented (Figure 2). These locus-specific discrepancies are 6/28 (21.4%) for HLA-A and 8/24 (33.3%) for HLA-B. In accordance with the expected data only rare antibody specificities directed against one or few antigens of the locus HLA-C(w) were available by virtual pre-selection. Three serum samples, however, were integrated. These are characterized by anti-Cw antibodies as the only anti-HLA class I antibodies which were combined only with additional anti-HLA class II antibodies not influencing the assay specific for anti-HLA class I DSA. 1/3 (33.3%) of the respective recipients did not detect virtual anti-Cw DSA although this number is generally too low to be adequately considerable. Divergent/undetected virtually positive results for the antigens of HLA-class II gene locus DR were calculated to be 3/29 (10.3%) and for DQ-antigens 8/31 (25.8%), respectively. Thus, apart from anti-Cw DSA due to their general lack between 24 and 31 combinations virtually defining DSA were investigated in order to detect DSA against antigens of the most important gene loci. The deviation rate from positive virtual results must, apart from the DSA indicating anti-DR DSA (10.3%), must therefore be regarded as unexpectedly high (21.4% to 33.3%) by highlighting that any fourth to any third DSA-specification of the 212 investigated donor-recipient combinations is not adequately recorded by this novel Luminex-based assay named DSA.

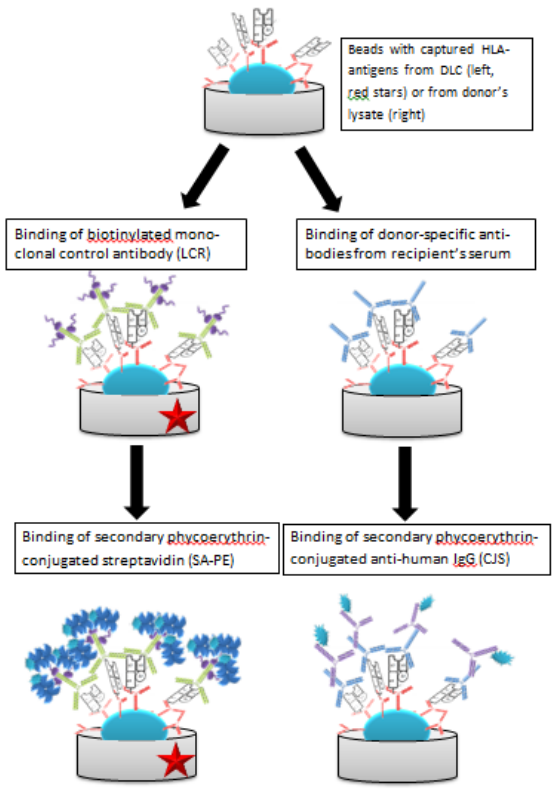

Figure 2: Flow scheme of the consecutive incubation steps in order to detect donor-specific antibodies from recipients' sera [right] or to obtain signals of biotinylated monoclonal control antibodies (LCR) [left]. The LCR positive control confirms the immobilization of a sufficient amount of HLA molecules by the capture antibodies in order to generate a signal. CJS (Conjugate Secondary): Phycoerythrin-conjugated goat anti-human IgG secondary antibody; LCR (Lysate Control Reagent): biotinylated monoclonal antibody directed against HLA-class I or II antigens, respectively; SA-PE: phycoerythrin-conjugated streptavidin; Red Stars: positive control using SA-PE (phycoerythrin-conjugated streptavidin) in the last incubation step in order to detect LCR (biotinylated monoclonal control antibodies).

Discussion

The requirement to substitute or at least to complement the conventional standard CDC-based crossmatch has increasingly been discussed over the last 10-12 years due to the various disadvantages described for this cell-based vitality assay. Already about 40 years ago Ozturk and Terasaki [24] reported that the CDC-crossmatch may be influenced by autoantibodies and immune complexes such as rheumatoid factors in a way leading to false-positive results. Cytotoxic autoantibodies were detected in patients suffering from autoimmune diseases such as systemic lupus erythematosus (SLE) without any previous alloimmunization. About twenty years later Sumitran-Holgersson [25] described falsified outcomes of CDC-based crossmatches as a consequence of autoantibodies and immune complexes as a frequent event. In order to avoid these diagnostic artefacts the reducing agents dithioerythritol/dithiothreitol (DTE/DTT) were used quite from the beginning of CDC-based cross-matching till this day in order to reduce the confusing influence of autoantibodies of the IgM isotype. However, as first described by Sumitran-Holgersson autoantibodies generated during autoimmune-mediated diseases such as SLE do not necessarily belong to the IgM isotype but may as well belong to the cytotoxic (i.e. complement-fixing) antibodies of the IgG (sub-) isotypes IgG1 and IgG3 [25]. Furthermore, there are several studies which point onto the detrimental effects of HLA-specific alloantibodies of the IgM isotype and thus clearly advise to detect and not to destroy these antibodies [26,27]. Unfortunately these IgM alloantibodies as well as so-called weak (low titer) IgG alloantibodies are eliminated using DTE/DTT although they may easily be detectable using solid phase crossmatch techniques modified with secondary anti-IgG/M antibodies [9,16]. These arguments have for years challenged the general diagnostic approach to use reducing agents in order to specify anti-HLA alloantibodies, and it has to be concluded that reducing agents are not at all applicable in order to selectively eliminate autoantibodies. Thus, autoimmune diseases especially of the immune complex type (type III) till this day represent a disruptive factor for CDC-based cross-matching which lead to an accumulation of these group of prospective recipients on the waiting lists for kidney allografts. On the one hand these diseases are frequently found as reasons for final renal failure, on the other hand they represent a common cause for artificially positive crossmatch outcomes raising this test to an unbreachable barrier for these prospective recipients [17,28,29].

Solid phase-based cross-matching, however, was first described in the context of CDC-based crossmatch interferences by therapeutic humanized monoclonal antibodies (moAb) [14]. Book and coworkers investigated CDC-based and flow cytometry-based crossmatch outcomes which were completely or at least partially manipulated by the application of rituximab (anti-CD20), basiliximab/Simulect (anti-CD25) and alemtuzumab/Campath (anti-CD52). Thereby they confirmed former investigations of Lyon et al. [30] and Wagenknecht et al. [31] regarding the administration of Campath. Book and coworkers first described the TMS-ELISA (those days GTI, today Immucor) as an adequate solid phase-based crossmatch tool in order to overcome the falsifying influence of those antibodies on CDC-based cross-matching. As they used the ELISA's old name TMS (Transplant Monitoring System) our group was unaware of their investigations for years up to 2013. Thus, the idea to implement a crossmatch assay in the context of AB0 bloodgroup-incompatible living kidney donations arose independently in our laboratory. The recipients were always pre-conditioned with anti-CD20 rituximab, which in all cases highly influenced the classical CDC-crossmatch leading to highly positive scores of 6 to 8 for B-cell cross-matching and scores between 2 and 4 for PBL cross-matching depending on individually varying fractions of B-cells. As rituximab belongs to the complement-activating isotype IgG1 its B-cell depleting activity and not that of HLA-specific alloantibodies was always monitored. Already in 2006 we implemented the downscaled second generation assay named Micro-AMS (Antibody Monitoring System) in our laboratory and for years used this system successfully in order to detect or exclude DSA in spite of the former application of the therapeutic antibodies rituximab and basiliximab [16,18]. Furthermore, the administration of cytostatic agents such as 6-Mercaptopurine in the context of haplo-identical hematopoetic stem cell donations which also falsified CDC-based pre-transplant cross-matching illustrated another field of our group's application of the Micro-AMS [16].

The fact that neither single nor vital cells are required as a given donor's material provided the opportunity to use the outer scleral rim of cornea donors which is generally available as retain sample after the excision of the inner part used for corneal allografting. Although this tissue is very poor in cells and these cells self-evidently cannot be isolated in order to be used in any vitality assay such as isolated lymphocytes, donors' corneal materials turned out to be adequate for the Micro-AMS-ELISA in order to predict forthcoming or to explain earlier corneal rejections [9,19]. The same aspect holds true for arterial vessel allografts characterized by very similar features of their tissue. Thus, the Micro-AMS was also successfully used in order to detect DSA directed against HLA-antigens of arterial allografts by our group [unpublished data].

Additionally, we provided for the first time data that the Micro-AMS and its follow-up assay, the AbCross-ELISA in its highly modified manner, was suitable to demonstrate the upcoming of donor-specific antibodies as a consequence of allografting using deep-frozen material (leukocyte pellets from spleen or peripheral blood) from deceased donors [32,33]. This was successfully done using donor's spleen derived leukocyte pellets which had been deep-frozen for 4.5 years. The procedure first provides the opportunity to systematically establish something like deceased donors' tissue banks thus having all of them available for this special application.

We here discuss various fields of application which clearly show the superiority of solid phase-based cross-matching over the CDC-based assay as this old-fashioned test generally does not meet many demands characterizing many recipients' individual situations. Thus, we suffered a hard setback when the Micro-AMS as a most reliable assay was suddenly discontinued by the manufacturer for mere commercial reasons in the year 2013. We had to establish the AbCross-ELISA (manufactured by MicroCoat, Bernried, Germany and distributed by Biorad, München, Germany) in a technically modified manner strongly deviating from the original protocol in a very short time span. According to the original protocol the binding of the recipient's donor-specific antibodies had to be fulfilled using intact lymphocytes of the respective donors. The resulting complexes of HLA-antigens and antibodies were afterwards isolated by a detergent-mediated lysis and those complexes immobilized to the HLA class II or class II specific capture antibodies, respectively. The resulting very laborious procedure including the initial density gradient centrifugation step in order to isolate lymphocytes, took about six hours. Furthermore, donors' blood volumes of about 20 ml in order to isolate sufficient numbers of HLA-class II antigens-expressing cells were required which have hardly ever been available from a given donor and least of all of a deceased donor. Thus, apart from circumventing artefacts which resulted from the artificial activation of the complement system monitored during CDC-cross-matching, the original AbCross-protocol did not provide any additional advantage over this cellular crossmatch variant. Many of the fields of application successfully performed using the Micro-AMS were not at all workable using the original AbCross-protocol by MicroCoat/Biorad which held true for all application fields dealing with donor materials lacking single or intact cells. As especially these donor materials justify the application of solid phase cross-matching the technical design of the AbCross-ELISA was completely changed and adapted to be in full accordance with the workflow of the Micro-AMS.

However, also the AbCross-ELISA was, again only for commercial reasons, discontinued by MicroCoat/Biorad at the end of 2016 leading us to search for a novel alternative. The information about the rebirth of the Micro-AMS obtained in those days seemed to be the solution of our problem. Using the same set of diagnostic antibodies, the Micro-AMS, now named Donor-Specific Antibodies/DSA, was unexpectedly manufactured again as a microbead-based array using the Luminex platform. With a view to establish this DSA-assay as the only remaining solid phase-based crossmatch system commercially available, it was systematically evaluated in our laboratory. However, the data provided by our present report are completely disappointing as the accordance between the virtual crossmatch results and corresponding DSA-based analyses is by far too low. Of the overall outcome comprising 212 positive and negative crossmatch results together 69 (32.5%) are discrepant. Of the whole number of 69 discrepant results of the DSA-assay 44 (64%) are divergent in a positive way in contrast to 25 results (36%) exhibiting data which diverge negatively from the virtually positive crossmatch results. Especially the high number of 44 deviations positively differing form negative virtual crossmatch results out of the 69 discrepant results must be regarded as puzzling. Although not only donor-specific but in nearly all cases no anti-HLA antibodies in general were virtually demonstrable for the respective HLA-class (Table 2), the respective DSA-analyses which were clearly positive lead to the hypothesis of being based on artefacts. Of course such an assumption of 44 donor-specific antibodies would lead to a refusal of an allocated organ in more than 40% of the respective patients although no donor-specific antibodies exist. Also the number of 25 negative deviations is too high to be acceptable as virtual donor-specific antibodies are not detected in about any fourth recipient (Table 2). Referring to the chosen cohort of recipients (n=106) instead of HLA class I or II-dependent single results, respectively, the number of discrepancies becomes dramatic: Not less than 62 (58.4%) of them are characterized by findings which are not at all supported by virtual cross-matching (Table 2).

It has already been mentioned above that due to the various aspects of carefully pre-selecting the underlying virtual antibody specificities the high error rate or rate of divergent results is in all likelihood not the consequence of erroneous virtual cross-matching. As the set of antibodies involved in the DSA-assay is the same as that used in Micro-AMS the immunochemical "hardware" may either not be the reason for the high error rate. As far as our experience goes there is a high probability that the problem primarily arose from insufficiencies of the software classifying the raw data as positive or negative for donor-specific antibodies. So-called background adjusted factors (BAF) are subtracted from the MFI raw value measured against the immobilized donor antigens. This BAF represents cut off values calculated considering the values of the above mentioned three control beads Con1 (albumin), Con2 (glycoprotein IV) and Con3 (naked bead) using a completely unknown (i.e. unpublished) equation which is specific for each lot. This subtraction leads to three so-called "adjusted MFI-values". Apparently a serum sample was classified as positive, if two of these three values were positive. Generally the significance of the value of the naked Con3-bead by its integration into the evaluation algorithm must critically be challenged. Furthermore, serum samples leading to increased values of all three control values were always classified as positive for DSA.

Conclusion

Thus, apart from the fact that the underlying evaluation software is something like a black box, values are apparently included which are senseless in consideration of immunological/ immunochemical aspects. Moreover, no clinical evaluation has apparently been

performed by the manufacturer as otherwise the described unacceptable deficiencies would have been revealed.

Finally it is noteworthy that the difficulties and insufficiencies of the DSA assay described here have very similarly been commented on by colleagues of three other HLA laboratories thus clearly demonstrating that the difficulties do not result from individual deficiencies of our laboratory. Taken together due to the considerable predictable harm for the patients through the use of the DSA-assay we cannot draw another conclusion than clearly recommend not to implement this assay in its current design.

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