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## Case Report: Anti-HLA-DQ $\alpha$ Antibodies Have Clinical Impacts in Highly Immunized Kidney Patients

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### Abstract

**Context and purpose:** Organ exchange programmes are important in order to ensure organs to highly immunized kidney patients. In this case report, we describe a highly immunized patient in an acceptable mismatch program who had an unexpected positive Complement-Dependent Cytotoxicity (CDC) crossmatch with a deceased donor exchange.

**Main findings:** Subsequent evaluation revealed donor specific antibodies characterized by serological epitopes, which included the HLA-DQ $\alpha$  chain, were masked in the standard IgG Luminex assay unless the serum was diluted; however, the antibodies demonstrated high MFI values when the serum was pretreated with EDTA or analyzed with the C1q assay. These findings led us to evaluate the extent of masked anti-HLA-DQ antibodies that included HLA-DQ $\alpha$ /DQ $\beta$  pairs rather than just the HLA-DQ $\beta$  chains in 16 highly immunized patients on the kidney transplantation waiting list at our transplantation center. We determined that 25% of the patients had antibodies directed towards serological epitopes that involved the anti-HLA-DQ $\alpha$  chain but were masked in the standard IgG assay.

**Principal conclusions:** We conclude that masked anti-HLA-DQ antibodies directed towards serological epitopes that also involves the HLA-DQ $\alpha$  chain are common and should be considered as they may cause a positive CDC crossmatch. Thus, donor specific HLA-DQ antibodies must be evaluated in relation to full HLA-DQ $\alpha$ ; DQ $\beta$  pairs in the serological molecules of the donor.

**Keywords:** HLA antibodies; Standard IgG single antigen bead assay; EDTA; C1q assay; Prozone effect

**Abbreviations:** CDC: Complement-Dependent Cytotoxicity; cPRA: Calculated Panel Reactive Antibodies; EDTA: Ethylenediaminetetraacetic Acid; FXM: Flow-Cytometric Crossmatch; HI: Highly Immunized; HLA: Human Leukocyte Antibodies; MFI: Mean Fluorescence Intensity; PBS: Phosphate-Buffered Saline; PCR-SSP: Polymerase Chain Reaction with Sequence-Specific Primer; PRA: Panel Reactive Antibodies; SAB: Single Antigen Bead; STAMP: Scandiatransplant Acceptable Mismatch Programme.

### Introduction

Donor specific human leukocyte antigen (HLA) alloantibodies (DSA) are risk factors for the development of rejection and decreased graft survival in kidney transplantation [1]. Highly sensitized kidney patients have a lower chance of receiving a donor kidney offer than other patients on waiting lists [2], which has motivated many transplantation centers to introduce special programs [2,3]. The basis of acceptable mismatch programs is the determination of unacceptable HLA antigen mismatches. Most programs consider only highly expressed HLA antigens encoded by HLA-A; B;DRB1, though this is supplemented by HLA-DQB1 in some centers [2,4]. The standard IgG Luminex single antigen bead (SAB) assay is widely used to tailor acceptable antigens for future organ offers. This approach is reasonable

as Luminex assays are considered to be at least as sensitive as the cellular based flow cytometric crossmatch (FXM) method and are much more sensitive than a crossmatch based on complement-dependent cytotoxicity (CDC) in antibody detection. However, the Luminex assays have revealed that transplanted patients frequently develop *de novo* antibodies directed against serological epitopes that involve lowly expressed HLA chains, such as HLA-DQ $\alpha$  and -DP $\beta$ , which is associated with poor graft prognosis [1] and challenging future transplantations. Additionally, a proportion of patient's exhibit inhibitory effects in their serum, leading to falsely reduced mean fluorescence intensity (MFI) levels in the IgG SAB assays, which is referred to as the prozone effect [5-7].

### Case

A 51-year-old highly immunized (HI) man with terminal kidney failure secondary to hypertension was accepted in the Scandiatransplant acceptable mismatch program (STAMP) [2] for HI patients in September 2014. He received an organ offer in February 2015 with an unexpected positive B-cell crossmatch in a CDC-based assay as HLA-A;B;C, DRB1 and DQB1 mismatches were all among the predefined acceptable antigens based on the analyses performed using a Luminex SAB assay (One Lambda Inc., Canoga/Los Angeles, CA). A positive B-cell crossmatch in the CDC-based assay is considered to be a contraindication for transplantation at our transplantation center, which is in line with international consensus guidelines [8]. The patient was temporarily inactivated in the STAMP, but remained on

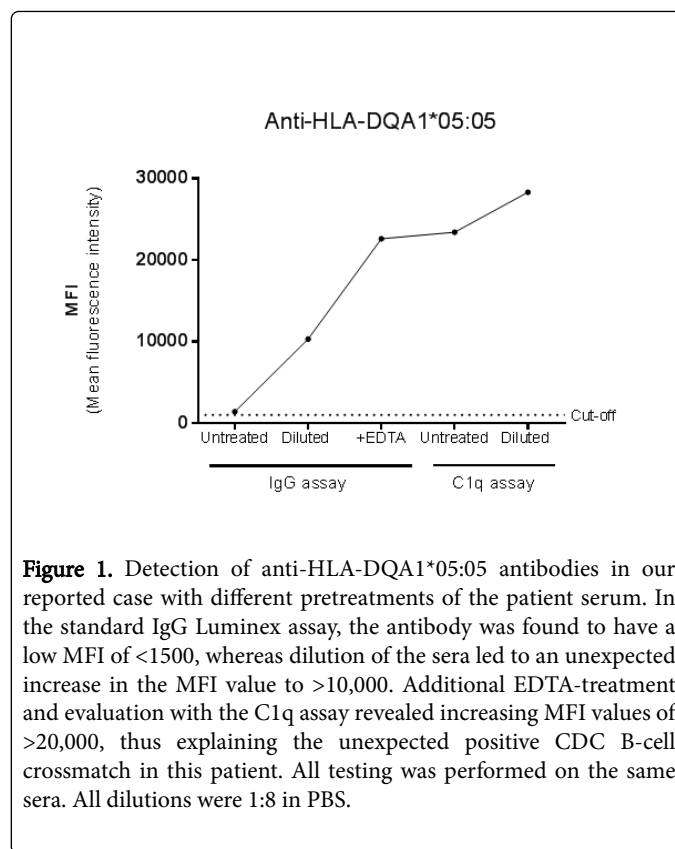
the local kidney transplantation waiting list during the review of his medical and immunological records.

**Medical history:** At the time of enrollment in the STAMP, the patient had a medical history of hemodialysis initiated in 1994 and previous kidney transplantation in July 1995 with a deceased donor. Due to a failing kidney graft, peritoneal dialysis was started in September 2010, and the patient was re-enrolled on the kidney waiting list in July 2011. When he entered the STAMP in September 2014, he had been on the waiting list for 38 months.

**Immunology:** The patient was HLA-A\*03,\*68; B\*35,\*38; C\*04,\*12; DRB1\*08:01P,\*13:01; DQA1\*01:03,\*03:01; DQB1\*03:02,\*06:03. Hospital records showed that the first graft in 1995 had introduced antigen mismatches at HLA-A1; B7,8; DR2,3; and DQ1. At the time of enrollment in the STAMP, the patient was HI, which is defined as having panel reactive antibodies (PRA)>80% in a CDC assay with DTT treated serum tested against a panel of B lymphocytes. Acceptable mismatches were defined by negative reactions (MFI<1000) in the Luminex SAB assay (One Lambda Inc., Canoga/Los Angeles, CA). Based on these analyses, the calculated PRA (cPRA) was 32% for HLA class I and 53% for HLA class II when the calculator provided by the HLA Fusion™ 2.0 software (version 3.3, One Lambda Inc., Canoga/Los Angeles, CA) was used against 1751 Danish donors as an approximation of the background population. The combined HLA class I and class II cPRA was 90% when a calculator provided by the ScandiTransplant database was used. None of these calculators considered antibodies directed against HLA-DQA1, DPA1 or DPB1. The patient had no antibodies directed against HLA-DP. Anti-HLA-DQ $\alpha$  antibodies were not considered to be a transplantation barrier as they were in linkage disequilibrium with HLA-DQB1 antigens that had already been excluded as acceptable antigens.

The organ offer from a deceased donor in the STAMP in February 2015 introduced HLA-A\*02,\*32; B\*40:01,\*44; C\*03:02,\*05; DRB1\*11; DQB1\*03:01 mismatches that were all among the predefined acceptable mismatches. The CDC crossmatch was highly positive against the donor B-cells but not against the donor T cells. Both analyses included DTT treated serum. HLA typing of the donor was confirmed at our center with real time PCR (LinkSeq, Linkage Bioscience, CA, US). Autoantibodies were excluded. We found that the patient reacted with an MFI>1000 for two out of five beads with HLA-DQB1\*03:01 antigens in combination with HLA-DQA1\*05:05 or HLA-DQA1\*06:01 but not in combination with HLA-DQA1\*02:01,\*03:01, or \*05:03. Among Caucasians, the HLA-DRB1\*11; DQB1\*03:01 haplotype is in linkage disequilibrium with HLA-DQA1\*05 (<http://www.allelefrequencies.net/hla6003a.asp>).

The donor was typed to be HLA-DQA1\*05:05 by polymerase chain reaction with sequence-specific primer (PCR-SSP) (Olerup SSP AB, Stockholm, Sweden). We investigated whether donor specific antibodies had been masked in the standard IgG Luminex bead assays, which were extended to include dilutions of serum and EDTA treated serum. Additionally, the LABScreen C1q SAB assay (One Lambda Inc., Canoga/Los Angeles, CA) was used with diluted and undiluted serum to evaluate complement binding. We found no additional anti-HLA-A, -Cw, -DRB1\*, -DRB3\*, -DRB4\*, -DRB5\*, -DQB1\* or -DP antibodies in these analyses. However, in the EDTA-treated sample, three additional anti-HLA-B antibodies were detected, though none of them were donor-specific and thus were unlikely to cause the positive B-cell crossmatch. Moreover, we found anti-HLA-DQA1\*05:05 antibodies with increasing MFI values in the diluted, EDTA-treated serum using the IgG assay as well as the C1q assay (Figure 1).



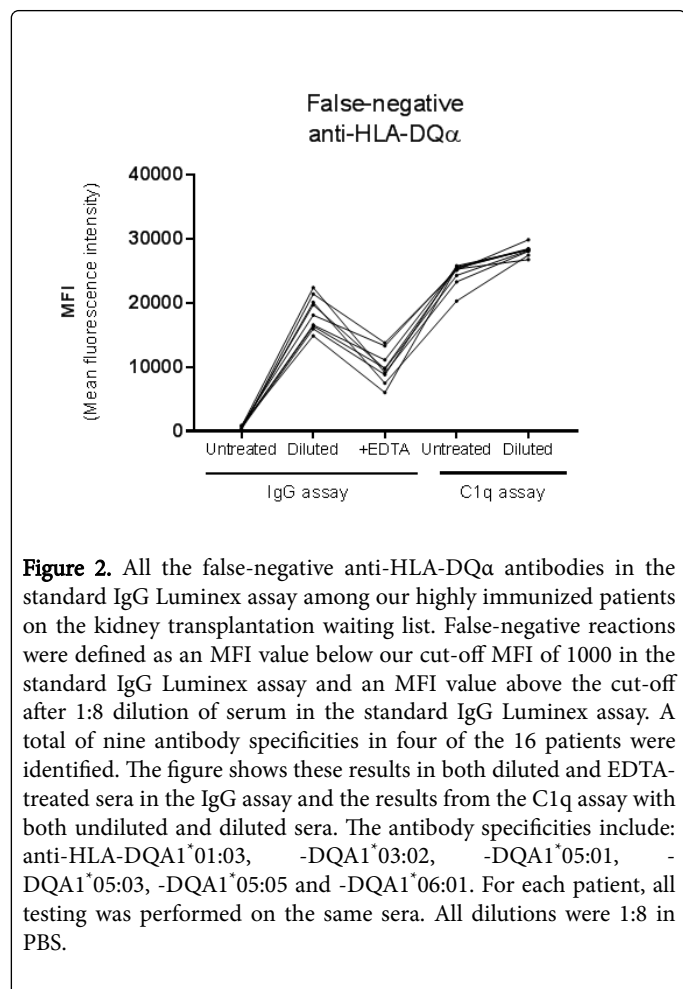
**Figure 1.** Detection of anti-HLA-DQA1\*05:05 antibodies in our reported case with different pretreatments of the patient serum. In the standard IgG Luminex assay, the antibody was found to have a low MFI of <1500, whereas dilution of the sera led to an unexpected increase in the MFI value to >10,000. Additional EDTA-treatment and evaluation with the C1q assay revealed increasing MFI values of >20,000, thus explaining the unexpected positive CDC B-cell crossmatch in this patient. All testing was performed on the same sera. All dilutions were 1:8 in PBS.

Similar patterns were found for anti-HLA-DQA1\*05:01 and anti-HLA-DQA1\*05:03. Beads with HLA-DQB1\*03:01 antigens remained negative in combination with HLA-DQA1\*02:01 and HLA-DQA1\*03:01 in all additional assays. Accordingly, we concluded that the positive B-cell crossmatch was most likely caused by complement binding/activating of the patient antibodies directed against serological epitopes that were encoded by HLA-DQA1\*05:05 alone or the HLA-DQA1\*05:05;DQB1\*03:01 pair. We performed additional dilution of a new serum and found an increase in the MFI value even when diluted 1:100 (data not shown). Thus, we found masked antibodies using the standard IgG Luminex SAB assay due to a prozone phenomenon caused by complement interference as they were unmasked after dilution or when EDTA treatment was used. Moreover, these antibodies were detected with the C1q assay, both in undiluted and 1:8 diluted sera, supporting that they had a high titer in addition to being complement binding.

**Follow-up:** In May 2016, the patient was re-enrolled in the STAMP with an exclusion of HLA-DQB1\*03:01 due to its linkage equilibrium to the HLA-DQA1\*05 encoded  $\alpha$ -chain. In July 2016, he received a kidney offer from a deceased donor with the following mismatches: HLA-A\*02,\*11; B\*15; C\*03:02,\*03:03 and DRB1\*04. The donor was a complete match for the HLA-DQ $\alpha$  and -DQ $\beta$  loci. The CDC B-cell crossmatch was negative, and the patient was transplanted.

To date, nine months after transplantation the graft is well-functioning without any diagnosed rejection episodes. The patient is normotensive without edema on 100 mg metoprolol and 20 mg furosemide. Plasma-creatinine has stabilized to approximately 180-200  $\mu$ mol/l and the glomerular filtration rate has been estimated to 40 ml/min, which is clinically satisfying for a transplanted kidney patient. Renal arterial resistance index was estimated at 0.7 at follow-up four

months after transplantation that is within the normal range. Additionally, the patient has a leukocyte count, plasma-calcium and plasma-phosphate within the normal range.

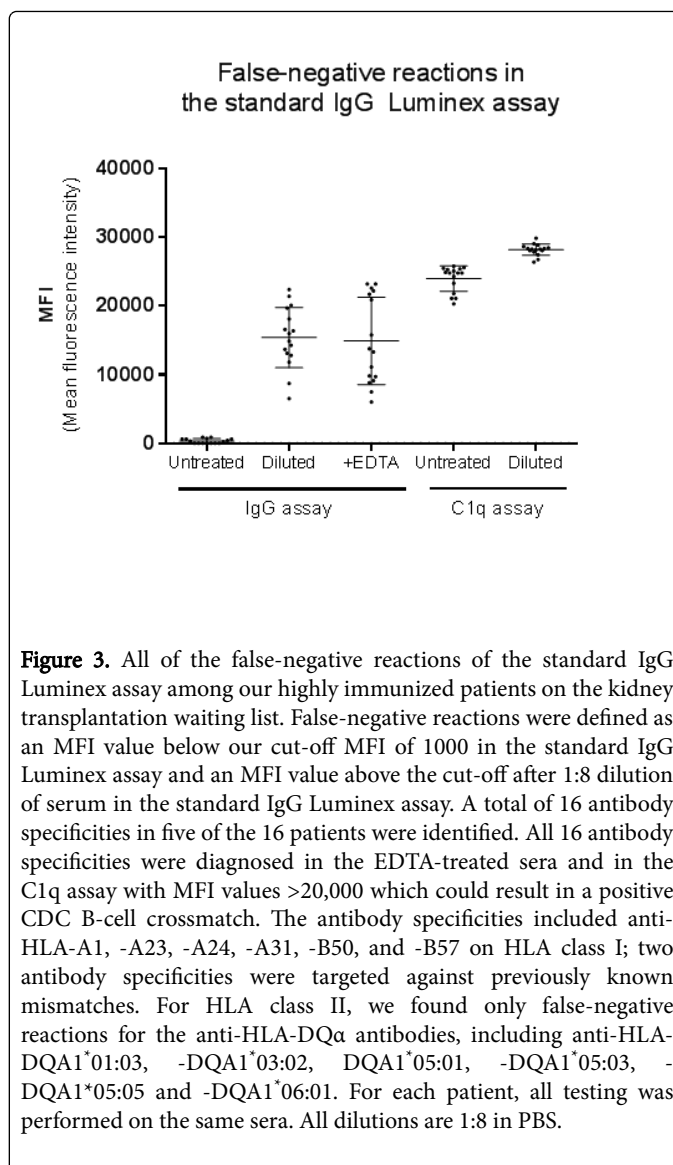


**Figure 2.** All the false-negative anti-HLA-DQ $\alpha$  antibodies in the standard IgG Luminex assay among our highly immunized patients on the kidney transplantation waiting list. False-negative reactions were defined as an MFI value below our cut-off MFI of 1000 in the standard IgG Luminex assay and an MFI value above the cut-off after 1:8 dilution of serum in the standard IgG Luminex assay. A total of nine antibody specificities in four of the 16 patients were identified. The figure shows these results in both diluted and EDTA-treated sera in the IgG assay and the results from the C1q assay with both undiluted and diluted sera. The antibody specificities include: anti-HLA-DQA1\*01:03, -DQA1\*03:02, -DQA1\*05:01, -DQA1\*05:03, -DQA1\*05:05 and -DQA1\*06:01. For each patient, all testing was performed on the same sera. All dilutions were 1:8 in PBS.

The interference of inhibitory factors in serum on the detection of antibodies directed towards HLA-DQ $\alpha/\beta$  epitopes.

Because of this case, we examined the extent of masked antibodies directed towards serological epitopes involving HLA-DQ $\alpha$  chains alone or HLA-DQ $\alpha/\beta$  pairs in 16 HI kidney patients on the waiting list using the standard IgG single antigen assay. HI was defined as having PRA>80% in a CDC-based screening against a B-cell panel. We performed testing with regular, 1:8 diluted and EDTA-treated patient sera in conjunction with testing using the C1q assay with regular and 1:8 diluted patient sera.

A total of four patients (25%) with nine antibody specificities, including our index patient, exhibited inhibition/prozone phenomena in the detection of antibodies directed towards epitopes on serological HLA-DQ $\alpha/\beta$  molecules. Inhibition was defined as an MFI<1000 in undiluted serum and more than double MFI in 1:8 diluted serum in the standard assay (Figure 2), and were thus classified as false-negative reactions for the standard Luminex IgG assay. All of these antibodies were detected after the addition of EDTA and in the C1q assay, both in undiluted and diluted serum, with MFI values above 20000, which may result in a positive CDC B-cell crossmatch, as occurred in the reported case.



**Figure 3.** All of the false-negative reactions of the standard IgG Luminex assay among our highly immunized patients on the kidney transplantation waiting list. False-negative reactions were defined as an MFI value below our cut-off MFI of 1000 in the standard IgG Luminex assay and an MFI value above the cut-off after 1:8 dilution of serum in the standard IgG Luminex assay. A total of 16 antibody specificities in five of the 16 patients were identified. All 16 antibody specificities were diagnosed in the EDTA-treated sera and in the C1q assay with MFI values >20,000 which could result in a positive CDC B-cell crossmatch. The antibody specificities included anti-HLA-A1, -A23, -A24, -A31, -B50, and -B57 on HLA class I; two antibody specificities were targeted against previously known mismatches. For HLA class II, we found only false-negative reactions for the anti-HLA-DQ $\alpha$  antibodies, including anti-HLA-DQA1\*01:03, -DQA1\*03:02, DQA1\*05:01, -DQA1\*05:03, -DQA1\*05:05 and -DQA1\*06:01. For each patient, all testing was performed on the same sera. All dilutions are 1:8 in PBS.

Our review was extended to include all identified anti-HLA antibodies, which revealed false-negative reactions in the standard IgG Luminex assay for a total of 16 antibody specificities among five patients (31%), including seven HLA-A and -B specificities and nine anti-HLA-DQ $\alpha$  antibodies (Figure 3) of which two (13%) had false-negative reactions corresponding to one or more previously identified HLA mismatches.

We observed a tendency of higher MFI values in diluted sera (1:8) compared to undiluted sera in the C1q assay (Figure 3), suggesting that prozone phenomena may occur in the C1q assay as well as in the IgG assay, although no false negative reactions were observed in the C1q assay.

## Discussion

In this case report, we found that antibodies directed towards serological HLA-DQ epitopes that involved the HLA-DQA1\*05:05 encoded  $\alpha$ -chain alone or the HLA-DQ $\alpha,\beta$  pair caused a positive CDC B-cell crossmatch in an HI kidney patient. These antibodies had a very

high MFI value in diluted, EDTA-treated sera and in the C1q assay, but they had a low MFI value in the standard IgG Luminex single antigen assay most likely due to complement interference, as the dissociation or destruction of complement C1 by EDTA treatment eliminated the effect as previously described [6,7]. There do not exist reports that address the importance of such antibodies in CDC crossmatching in the pre-transplantation setting as the HLA-DQ $\alpha$  chains is not evaluated in most clinical settings. One report described a patient who developed anti-HLA-DQ $\alpha$  antibodies post-transplantation [9] and two cases reported unexpected positive B-cell FXM due to donor specific anti-HLA-DQ $\alpha$  antibodies in living donor kidney transplantations [10,11]. This case underlines both 1) the possible clinical significance of antibodies directed towards serological epitopes that involves the HLA-DQA1 encoded  $\alpha$ -chain in the HLA-DQ molecule in the pre-transplantation setting and 2) the common occurrence of masking of such antibodies in HI kidney recipients.

The majority of *de novo* DSA are directed against HLA class II antigens, especially serologically defined HLA-DQ antigens mainly defined by HLA-DQB1, as reviewed by Filippone et al. [1]. This approach may result in an underreported incidence of antibodies directed towards epitopes that involve the HLA-DQ $\alpha$  chain as well, and thus an underestimation of the clinical consequence of these antibodies, when previously transplanted patients undergo future transplantation [12,13]. The antibody reactions in our patient correspond to the DQA 41GR eplet and our findings support that the current nomenclature system does not reflect the true nature of HLA-DQ polymorphisms as concluded by Tambur et al. [14]. Currently, the typing of HLA-DQ $\alpha$  chains in donors and the reporting of anti-HLA-DQ $\alpha$  antibodies in recipients are not mandatory in many clinical programs such as STAMP in our center, although our case demonstrates the potential problems of positive crossmatching [12]. Another concern is the interpretation of HLA-DQ antibodies when DQ $\alpha$ - and DQ $\beta$ -chains are paired on beads, which may block the DQ $\alpha$  epitopes of the molecule, reducing the significance of their contribution [13]. We found false-negative antibodies directed towards epitopes that involved the HLA-DQA1 encoded  $\alpha$ -chain in 25% of the HI patients in our cohort. We suggest that the clinical consequences of these antibodies are underestimated and prospective HLA-DQA1 typing combined with HLA-DQB1 of kidney donors should be performed when antibodies directed towards HLA-DQ molecules are evaluated. Moreover, acceptable HLA mismatches have previously often been solely based on findings from the standard IgG SAB assay for highly immunized patients but we found that 31% had false-negative reactions due to the effect of inhibitory factors in serum and that 13% had false-negative reactions corresponding to one or more previously identified HLA mismatch in our HI patients.

## Conclusion

We report that antibodies directed towards epitopes that involve the HLA-DQA1 chain in HI patients are common but often overlooked, which may result in a positive CDC B-cell crossmatch as these antibodies often bind C1q according to our investigation. Additionally,

we conclude that donor specific HLA-DQ antibodies must be evaluated in relation to full HLA-DQ $\alpha$ ; DQ $\beta$  pairs in the serological molecules of the donor.

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