

# **Evaluation of Two New Antibody Detection Techniques in Kidney Transplantation**

Doctoral Thesis

**Dr. Petra Gombos**

Semmelweis University  
Doctoral School of Pathology



**Supervisor:**

Dr. Róbert Langer, Ph.D.

**Consultant:**

Dr. Caner Süsal

**Official reviewers:**

Dr. Péter Sótónyi, Ph.D.

Dr. Zsolt Káposztás, Ph.D.

**Ph.D. Final Examination Board Chair:**

Dr. Zoltán Szabó, Ph.D.

**Ph.D. Final Examination Board:**

Dr. László Szőnyi, Ph.D.

Dr. Krisztina Czebe, Ph.D.

Budapest

2014

## **1. INTRODUCTION**

While in the new immunosuppression era, graft damage caused by acute cellular rejection has almost disappeared, due to the increasing number of human leukocyte antigen (HLA)-mismatched transplantations and sensitization of the recipients at B-cell level, antibody-mediated rejection (AMR) still remains a problem. Therefore, prevention of AMR became an important issue in the field of kidney transplantation.

In the pathogenesis of AMR complement activation caused by preformed donor-specific HLA immunoglobulin (Ig) G antibodies (DSA) play a critical role. To protect graft function, a combination of different diagnostic measures, such as HLA-typing, crossmatch (XM) and antibody screening is needed.

XM techniques are assays to identify the presence of preformed DSA against donor HLA class I and II antigens in the serum of recipients before transplantation on the kidney transplant waiting list. For crossmatching, the recipient's serum and donor lymphocytes have to be available.

To avoid a positive XM in the transplant centers and thus prevent AMR, kidney transplant recipients are screened periodically for the presence of HLA antibodies before transplantation to define the „unacceptable HLA antigen mismatches“. For the detection of DSA, serum of the recipient

has to be available, but instead of the lymphocytes of potential donor, a panel of HLA-typed lymphocytes from healthy blood donors, their solubilized HLA antigens or artificially produced recombinant HLA molecule panels are utilized.

Different assays for identification of HLA antibodies vary in the type of target, format, sensitivity and specificity. Assay targets can either be cells tested for example in cytotoxicity assay, such as complement-dependent cytotoxicity assay (CDC) or soluble antigens tested in solid-phase immunoassays such as enzyme-linked immunosorbent assay (ELISA) and Luminex single antigen bead (SAB) technique. With increasing sensitivity of the diagnostic assays, weak DSA can be detected appropriately in serum of recipients on the kidney transplant waiting list. While CDC assay can detect from very high to high or moderate DSA levels, ELISA assay is more sensitive to determine moderate or low antibody reactivity in the recipient's serum. Nowadays, Luminex SAB is the only methodology with its high sensitivity, which can detect low titer DSA with high accuracy.

Currently it is a matter of debate, which antibody test at what sensitivity should be used in the pretransplant evaluation of alloantibodies before kidney transplantation.

## **2. THE AIMS OF THE THESIS**

False positive results as well as low sensitivity can create difficulties in the correct assessment of the patient's HLA antibody status on the kidney transplant waiting list. We analyzed the advantages and the problems associated with two recently introduced HLA antibody detection methods.

In the first study we investigated the potential superiority of the commercially available AbCross<sup>®</sup> ELISA XM over the B-cell CDC XM (BXM) in predicting graft loss. Because there is debate about the sensitivity and clinical relevance of the BXM in renal transplantation, we analyzed, whether with the new AbCross<sup>®</sup> technique the disadvantages of the BXM, such as the detection of unspecific reactions or autoantibodies, can be eliminated.

In the second study, to estimate the impact of the problem of potentially "false positive" results detected with the highly sensitive Luminex SAB technique on the sensitization status of patients on the kidney transplant waiting list, we investigated the prevalence of HLA antibodies in waiting list patients of the Heidelberg transplant center using three different assays, namely CDC T-cell screening, AbScreen<sup>®</sup> ELISA screening, and SAB in parallel. A high prevalence of HLA antibody reactivity with a given assay in patients without any history of

immunization would indicate that this particular assay generates false positive results. We also examined in detail the HLA specificity and strength of the false positive reactions. Such information could be useful in the daily routine, when SAB results are evaluated in the individual patients.

## **2.1 Comparison of the clinical relevance of ELISA and B-cell CDC crossmatch before kidney transplantation**

The following questions were addressed:

- a. What is the rate of 2-year graft loss after kidney transplantation in AbCross<sup>®</sup> ELISA XM-positive and AbCross<sup>®</sup> ELISA XM-negative patients?
- b. What is the rate of 2-year graft loss after kidney transplantation in BXM-positive patients compared to BXM-negative patients?
- c. Is the impact of positivity in AbCross<sup>®</sup> ELISA XM on graft survival supported by AbScreen<sup>®</sup> ELISA screening results?

- d. Is there a relationship between kidney graft survival and BXM and AbCross<sup>®</sup> ELISA XM or AbScreen<sup>®</sup> ELISA screening results?

## **2.2 Evaluation of the influence of the recently introduced Luminex SAB on the sensitization status of patients on the kidney transplant waiting list**

In this context, the following questions were addressed:

- a. What is the prevalence of the positive patients on the kidney transplant waiting list in the SAB technique compared to the less sensitive ELISA and CDC methods?
- b. What is the prevalence of HLA antibody-positive patients without any immunization history?
- c. Which mean fluorescence intensity (MFI) values have patients without any history of immunization?
- d. Whether the problem of false positive results could be solved by increasing the cutoff values?
- e. What is the prevalence of SAB-positive patients according to reaction with the percentage of beads?
- f. Which HLA allele specificities react positive in SAB in patients without history of immunization?

- g. Whether other methods, such as another vendor or another test principle, can solve the problem of false positive results?

### **3. PATIENTS AND METHODS**

#### **3.1 Patients**

In the first study in which the potential superiority of the ELISA XM over the BXM before kidney transplantation was evaluated, pretransplant sera of 271 living or deceased donor kidney transplant recipients who were transplanted at the Heidelberg transplant center between 1998 and 2010 and on whom frozen donor cell material was available were tested in the AbScreen<sup>®</sup> ELISA screening assay for the presence of HLA antibodies and in BXM and AbCross<sup>®</sup> ELISA XM assays for antibody reactivity against donor B-cells or donor HLA class I and II antigens, respectively.

In the second study in which the influence of the Luminex SAB test on the sensitization status of patients on the waiting list was evaluated in parallel with the ELISA and CDC screening methods, pretransplant sera of 534 patients on the Heidelberg kidney transplant waiting list were additionally analyzed using the SAB assay.

## **3.2 Methods**

### **3.2.1 Study 1: Comparison of the clinical relevance of ELISA and B-cell CDC crossmatch before kidney transplantation**

In the first study, for the CDC XM, the donor's separated B-lymphocytes were used and the cytotoxicity effect was examined using a fluorescent microscope (Leica, Wetzlar, Germany). Cell death >20% was considered positive.

In addition, sera were tested using the recently introduced AbCross<sup>®</sup> ELISA XM (BioRad, Munich, Germany) assay, in which solubilized donor HLA molecules are used to detect DSA. AbCross<sup>®</sup> ELISA XM is a commercially available solid phase XM technique with advantages over the standard BXM, such as higher reproducibility, objectivity, sensitivity and specificity for HLA antigens. The AbCross<sup>®</sup> ELISA XM assay detects antibodies on the microtiter plate coated with monoclonal antibodies. The results were detected with photometric measurement in an ELISA reader and optical density (OD) greater than or equal to the double of the negative control were considered positive.

The sera were also tested for the presence of IgG-anti-HLA class I and II alloantibodies using AbScreen<sup>®</sup> ELISA (BioRad) kits, which use pooled HLA molecules on 96-well microtiter



plates for the detection of HLA antibodies. HLA antibodies of the recipient are determined on separate plates against pooled class I or class II HLA molecules. Based on previous clinical findings, an OD of  $\geq 0.300$  was used as cutoff for anti-HLA positivity.

Two-year clinical follow-up data were collected and documented for 223 of 271 patients and statistical analysis was performed using the chi-square test.

### **3.2.2 Study 2: Evaluation of the influence of the recently introduced Luminex SAB on the sensitization status of patients on the kidney transplant waiting list**

In the second study, the different antibody screening techniques were analyzed. At the Heidelberg transplant center, waiting list patients are routinely screened every three months for HLA antibodies employing ELISA and CDC. In addition, the 534 sera from the third quarter of 2010 were examined using the SAB method.

Panel reactive antibody (PRA) against total lymphocytes (mainly T-cells) of a panel of 56 cell donors on frozen/thawed cell trays were determined using the CDC method in the absence of DTT (<http://www.ctstransplant.org/public/reagents/seroCell.shtml>).

Following standard procedure, the patient's serum was incubated with lymphocytes, complement was added and the trays were read using a fluorescent microscope (Leica, Wetzlar, Germany). PRA of >5% was considered positive.

Furthermore, all 534 sera were tested for the presence of HLA class I and II alloantibodies using AbScreen<sup>®</sup> ELISA kits of BioRad (Munich, Germany), which as mentioned already above, utilize pooled HLA molecules attached to microtiter plates and enable the detection of HLA-A, -B, -C, -DR, and -DQ antibodies of the IgG isotype. Based on previous clinical findings, an OD of  $\geq 0.300$  was used as cutoff for anti-HLA positivity in ELISA. In one patient who was negative with SAB but positive by ELISA AbScreen<sup>®</sup>, the ELISA-PRA assay (AbIdent<sup>®</sup>, BioRad, Munich, Germany) which utilizes cell lysates from single individuals instead of pooled lysates was used to confirm the absence of HLA antibodies.

In addition, all sera were tested using the LABScreen<sup>®</sup> Luminex kits of One Lambda (Canoga Park CA, USA, LS1A04 Lot006 and LS2A01 Lot008), using SAB-coated beads that enable the identification of IgG antibody specificities against HLA-A, -B, -C, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1 and -DPB1. Because no standard cutoff for the SAB assay is recommended by the manufacturer, the value of

MFI  $\geq$ 1,000, which has been commonly indicated in the literature, was used as a cutoff.

Sera of 10 male waiting list patients without a history of immunization, who were positive in the LabScreen<sup>®</sup> SAB test against HLA alleles that are rather common in the general population, were tested subsequently in the Lifecodes<sup>®</sup> SAB (Gene-Probe Transplant Diagnostics, Lifecodes<sup>®</sup> LSA, Stamford, CT) assay, in which positivity is defined by the software of the manufacturer when two of the three standard calculation values are over the predetermined cutoff.

Furthermore, sera defined as positive by the LabScreen<sup>®</sup> SAB test of 20 male waiting list patients without a history of immunization and 15 non-immunized male healthy blood donors with unknown previous LabScreen<sup>®</sup> SAB results, were tested in the LabScreen<sup>®</sup> PRA assay (One Lambda), which utilizes 55 beads coated with HLA antigens purified from 55 different human cell lines (phenotype panel). The cutoff for positivity was set at 1,000 MFI.

Clinical background data including transfusions, pregnancies and previous transplantations were requested from the patient's clinical care facilities. Fisher's exact test was used for statistical comparison.

## **4. RESULTS**

### **4.1 Comparison of the clinical relevance of ELISA and B-cell CDC crossmatch before kidney transplantation**

To our questions the following answers were found:

- a. The 2-year graft loss rate in 37 recipients positive for DSA in AbCross<sup>®</sup> against donor HLA class I or II antigens was 19%, which is significantly higher than the 8% rate in 186 recipients who were negative for both HLA antibody classes in AbCross<sup>®</sup> ( $P=0.043$ ).
- b. Within 2 years after transplantation, the rate of graft loss in 14 CDC BXM-positive patients was 7%, not higher than the 9% rate in 206 CDC BXM-negative patients ( $P=0.79$ ).
- c. Corresponding with the AbCross<sup>®</sup> ELISA XM results, 48 patients positive for HLA class I or II antibodies on ELISA screening had at 2 years a significantly poorer graft outcome than 174 recipients who were negative for HLA class I and II antibodies (graft loss rate, 21% vs. 6%;  $P=0.002$ ).
- d. When CDC BXM was analyzed in combination with the AbCross<sup>®</sup> ELISA XM, the rate of graft loss 2 years

posttransplantation in 34 BXM-negative but AbCross<sup>®</sup>-positive patients was 21% compared with 7% in 172 BXM- and AbCross<sup>®</sup>-negative patients ( $P=0.012$ ), and 9% in 11 BXM-positive but AbCross<sup>®</sup>-negative patients ( $P=0.39$ ). The low number of BXM-positive and AbCross<sup>®</sup>-positive patients did not allow a meaningful analysis ( $n=3$ ; 2-year graft loss rate, 0%). When CDC BXM was analyzed in combination with ELISA screening, the rate of graft loss at 2 years after transplantation in 44 BXM-negative but AbScreen<sup>®</sup>-positive patients was 21%, significantly higher than the 6% rate in 162 BXM-negative and AbScreen<sup>®</sup>-negative patients ( $P=0.002$ ) and higher than the 0% rate in 9 BXM-positive but AbScreen<sup>®</sup>-negative patients ( $P=0.14$ ).

#### **4.2 Evaluation of the influence of the recently introduced Luminex SAB on the sensitization status of patients on the kidney transplant waiting list**

To our questions the following answers were found:

- a. When all 534 patients on the waiting list were analyzed, 5% ( $n=28$ ) were positive for HLA antibodies in CDC, 14% ( $n=73$ ) in ELISA screening and 81% ( $n=435$ ) in SAB.

- b. Medical records and patient interviews indicated that 133 of these patients (32%) had not been exposed to any immunizing event, such as blood transfusions, pregnancies or previous transplantations. Only one of the 133 patients (1%) was positive in the ELISA screening test for HLA class II, all were negative in ELISA for HLA class I, and two patients were positive in CDC (2%). In contrast to these CDC and ELISA results, as many as 77% (n=102) of the patients without a history of immunization were found to possess HLA antibodies using SAB.
- c. At a cutoff of 2,000 MFI, 50% of the non-immunized patients were HLA antibody positive, and at a cutoff of 5,000 MFI 25% of these patients were positive, showing that “false positive” reactions in the SAB assay were not restricted to “weak” reactions.
- d. Some of these antibodies reacted quite strongly, with MFI values up to 14,440, so that raising the reactivity cutoff did not eliminate the problem associated with these false positive reactions.
- e. Sera of CDC- and ELISA-negative patients without a history of immunization showed a restricted SAB reactivity pattern and reacted in 86% of the cases with

≤5% of the SABs. In contrast, 94% of the ELISA- or CDC-positive patients showed positive reactions against >5% of the SABs.

- f. Some patients without a history of sensitization had antibodies with high MFI values against HLA specificities that are rather common in the general population, such as A\*24:02 (prevalence in this series: 8.8%, maximum MFI: 12,197), B\*08:01 (7.8%, MFI: 9,862), B\*44:02 (7.8%, MFI: 10,427) or C\*05:01 (7.8%, MFI: 3,962) (8.7%, 12.5%, 9.0%, 9.1% population prevalence, respectively). Similarly, among patients with antibodies against HLA class II, some had antibodies against beads carrying DQB alleles that are rather common, such as DQB1\*03:01 (prevalence in this series: 7.8%, MFI: 9,804), which occurs at a frequency of 18.5% in the general population.
- g. When the more common HLA specificities were analyzed, 8 of the 10 patients did not show HLA antibody reactivity in the SAB assay of the second vendor whereas 2 of the 10 did. When 1,000 MFI was used as cutoff for positivity, 6 (30%) of the 20 non-immunized male waiting list patients who were positive in SAB testing were also positive in the LabScreen<sup>®</sup> PRA test. None of the 15 healthy male blood

donors was positive in this test whereas 9 of them had been shown positive in the LabScreen<sup>®</sup> SAB assay with reactivities ranging from 1,011 to 4,424 MFI against 21 different HLA alleles, among them B\*44:02 which occurs in more than 7% of Caucasians.

## **5. CONCLUSIONS**

Our data indicate that the AbCross<sup>®</sup> ELISA XM is superior to the BXM, most likely because it detects antibodies against donor HLA antigens at a higher sensitivity.

Refusal of donor kidneys to recipients based on HLA antibody specificities detected “exclusively” in the SAB assay is not advisable. False SAB reactions can be unveiled by pretesting with additional antibody assays.



## **6. LIST OF PUBLICATIONS**

### **6.1 Publications related to the thesis**

- 1. Gombos P**, Opelz G, Scherer S, Morath C, Zeier M, Schemmer P, Süsal C. (2013) Influence of test technique on sensitization status of patients on the kidney transplant waiting list. *Am J Transplant*, 13: 2075-2082. **(IF 6.192)**
- 2. Gombos P**, Opelz G, Scherer S, Morath C, Zeier M, Schemmer P, Langer RM, Süsal C. (2013) Superiority of AbCross Enzyme-Linked Immunosorbent Assay Cross-Match Over the B-Cell Complement-Dependent Lymphocytotoxicity Cross-Match. *Transplant Proc*, 45: 1383-1385. **(IF 0.952)**

### **6.2 Additional publications**

- Opelz G, Döhler B, Ruhstroth A, Cinca S, Unterrainer C, Stricker L, Scherer S, **Gombos P**, Süsal C, Daniel V, Tran H. (2013) The collaborative transplant study registry. *Transplant Rev*, 27: 43-45. **(IF 2.675)**
- Schaefer B, Tönshoff B, Schmidt J, Golriz M, Mehrabi A, **Gombos P**, Morath C, Wühl E, Schaefer F, Schmitt CP. (2013) Bleeding complications in pediatric ABO-

incompatible kidney transplantation. *Pediatr Nephrol*, 28: 327-332. **(IF 2.939)**

3. **Gombos P**, Langer RM, Korbely R, Varga M, Kaposi A, Dinya E, Müller V. (2010) Smoking following renal transplantation in Hungary and its possible deleterious effect on renal graft function. *Transplant Proc*, 42: 2357-2359. **(IF 0.993)**
4. Süsal C, Wettstein D, Döhler B, Ruhenstroth A, Scherer S, Tran H, **Gombos P**, Morath C, Schemmer P, Weimer R, Norman D, Bösmüller C, Slavcev A, Zivcic-Cosic S, Wagner E, Zeier M, Opelz G. Association of Kidney Graft Loss With Posttransplant Presence of HLA Antibodies Detected by Single Antigen Testing. **(in preparation)**