Format for ANSWERING REVIEWERS

June 9, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: ESPS Manuscript No: 10249-review.doc).
I would like to thank the reviewers for carefully reading the manuscript and providing constructive comments that have improved the manuscript.

Title: METHODOLOGICAL ASPECTS OF ANTI-HLA ANTIBODY ANALYSIS IN SOLID ORGAN TRANSPLANTATION
A review of the literature

Author: Andrew L. Lobashevsky

Name of Journal: *World Journal of Transplantation*

ESPS Manuscript NO: 10249

The manuscript has been improved according to the suggestions of reviewers:

Item-by-item response to comments from reviewer **#00504335**:

1. I have deleted all personal communication references.

2. I have revised the figures and figure legends, and I have made changes (in italic font) according to reviewer's suggestions.

3. The reviewer's comment regarding "enhancing antibodies" is addressed as follows:

   There are several reports regarding allograft protective gamma-globulins extracted from retroplacental units. Viosin GA used mice with mismatched class I MHCs to demonstrate inhibition of mixed lymphocyte reaction (MLR) [Viosin GA. Immunology Understood through Pregnancy. American Journal of Reproductive Immunology. 1998, v.40, p.124-129]. In addition, Riggio RR et al. reported considerable enhancement of kidney allograft survival in the recipients treated with gamma-globulin retroplacental extract. However, these researchers did not detect correlations between the degrees of donor-recipient HLA mismatch and the efficacy of RDGG treatment [Riggio RR, et al., Enhanced kidney graft survival with retroplacental source γ-globulin. Transplantation. 9182, v.33, p.636]. Moreover, the specificity of "antibodies" in placental extracts was not determined. These investigators speculated that RPGG might contain antibodies against MHC loci including DR. The proposed protective mechanism was related to formation of anti-idiotypic antibodies. The current review addresses the methodological aspects of detecting any anti-HLA antibodies. The author believes that the concept of protective antibodies is beyond the scope of the report.

Item-by – item response to reviewer **#0006809**.

1. I would like to stress that the purpose of this review is to familiarize the reader (preferably in the area of clinical transplantation) with the contemporary methodological aspects and pitfalls of anti-HLA antibody analysis in solid organ transplantation. However, the review does not specifically address the issue of non-HLA antibodies such as anti-endothelial cell antibodies, anti-MICA, and angiotensin-II receptor 1 antibodies. **The author agrees with the reviewer that the pre-transplant presence of such antibodies increases the risk of graft failure, but this issue is beyond the scope of the review.**

2. The issue of antibodies to denatured/cryptic epitopes and their significance has been addressed in the review (see page #8 and Figure 3). There are approximately 2500 solid phase antibody tests performed in my laboratory for kidney, liver, pancreas, lung, and heart transplant patients (pre- and post-transplant) annually. These include IgG and C1q assays. Usually, antibody analysis is accompanied by a flow cytometry cross-match assay performed prospectively or retrospectively. Anti-class I antibodies to denatured/cryptic epitopes have only been detected in four cases when the single antigen bead test was positive and the flow cross match results were negative. In such situations, two additional tests are performed: acid denaturing analysis (reagent from One Lambda) and flow cytometry cross match testing with lymphocytes from a surrogate donor with an HLA phenotype similar to the actual donor. Negative flow cross match results and positive acid denaturing tests allow us to conclusively determine the presence of antibodies to denatured/cryptic epitopes. The iBead method mentioned by the reviewer is no longer in use because One lambda discontinued its production. **The author agrees with the reviewer's point regarding effects of antibodies to cryptic/denatured antigens on cPRA and virtual cross match results**. Virtual cross match for lung and heart recipients has been used at our transplant center since 2008. In addition to solid phase antibody analysis, serum samples from these patients are tested against surrogate donors monthly/quarterly. No discrepancies between cross match results and single antigen solid phase analysis have been observed, except for the cases mentioned above. The author does not fully understand of the following statement by the reviewer, "**In this study WE describe identification of antibodies to cryptic HLA present on denatured forms of HLA on single antigen bead array and provide a reassessment of calculated panel-reactive antibody (CPRA) based on elimination of false-positive reactions due to antibodies to cryptic HLA epitopes, to identify antibodies to cryptic HLA vs native HLA".** This comment is very confusing because it looks like the reviewer copied it from another paper and pasted it into the comments. It is also unclear what "WE" is referencing. There is a paper published in The Journal of Heart and Lung Transplantation entitled "Practical value of identifying antibodies to cryptic HLA epitopes in cardiac transplantation" in 2014 (volume and pages have not been assigned yet, but available online at http://dx.doi.org/10.1016/j.healun.2014.02.013). The authors of this report indicate that antibodies to cryptic epitopes were identified in 20.8% of their heart transplant candidates and in 4.9% of antibodies analyzed.
3. The reviewer's statement "As a clinical diagnostic, single antigen bead assays are widely used to screen for HLA-specific antibodies in patient sera. Such assays are very effective at determining reactivity to a given HLA allotype, but it remains

difficult to determine antibody concentration or functional relevance via gradations in MFI" also requires considerable clarification. First, the author understands that solid phase antibody analysis is a very sensitive and specific test, but it is still semi-quantitative for determining antibody concentration. Secondly, one of the purposes of the solid phase antibody analysis, as specified on the page 7, Figure 1, and Figure 2, was to detect correlations between antibody relevance (MFI values) in DTT (generally used reducing agent that destroys IgM antibodies) treated serum and the median channel shift of flow cytometry cross match test. A correlation analysis was performed and addressed in the review. In addition, reports from other laboratories have shown similar findings (see multiple reports of A. Zacharie, etc). Third, I do not believe that SPR testing could be used as a part of the routine transplant work-up. In addition, SPR transducers are usually constructed by using prism coupling of incident light onto an optical substrate that is coated with a semitransparent noble metal. However, beads are made of latex/polystyrene. Lastly, the behavior of antibodies (at the nano level) while interacting with HLA proteins attached to the beads also strongly depends on the shape of the substrate, i.e., oval, rectangle, oblong, etc.

4. **The author agrees with the reviewer that cross-match results (MCS) and MFI values cannot be directly converted into antibody titers.** However, MFI values of donor specific antibodies (see above and page 7 in the paper) can be used as a reliable predictor of positive cross-match and graft outcome (MA Mujtaba, W Goggins, **AL Lobashevsky** et al. The strength of a donor-specific antibody is a more reliable predictor of antibody-mediated rejection than flow cytometry cross match analysis in desensitized kidney recipients. Clin Transplantation. 2011, 25(1), 96-102; A. Mujtaba, T. Taber, **A. Lobashevsky e**t al., Early findings of Prospective Anti-HLA donor specific Antibodies Monitoring study in pancreas transplantation. Indiana University Health Experience. Clin Transplant. 2012 Sep-Oct;26(5):E492-9).

Item-by – item response to reviewer **#00503180**

Comments regarding language:

All the suggestions given by the reviewer have been addressed.

Item-by – item response to reviewer **#0050625**

1. All the suggestions given by the reviewer regarding typos in "Non-specific antibody reactivity" have been addressed.
2. Four references from 2014 have been added to the manuscript.

Sincerely yours,

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