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Comparative Assessment of Anti-HLA Antibodies Using Two Commercially Available Luminex-Based Assays

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Background. Allospecific anti-HLA antibodies (Abs) are associated with rejection of solid organ grafts. The 2 main kits to detect anti-HLA Ab in patient serum are commercialized by Immucor and One Lambda/ThermoFisher. We sought to compare the performance of both platforms. **Methods.** Background-adjusted mean fluorescence intensity (MFI) values were used from both platforms to compare sera collected from 125 pretransplant and posttransplant heart and lung transplant recipients. **Results.** Most HLA class I (94.5%) and HLA class II (89%) Abs with moderate to high MFI titer (≥4000) were detected by both assays. A modest correlation was observed between MFI values obtained from the 2 assays for both class I ($r = 0.3$, $r^2 = 0.09$, $P < 0.0001$) and class II Ab ($r = 0.707$, $r^2 = 0.5$, $P < 0.0001$). Both assays detected anti–class I and II Ab that the other did not; however, no specific HLA allele was detected preferentially by either of the 2 assays. For a limited number of discrepant sera, dilution resulted in comparable reactivity profiles between the 2 platforms. **Conclusions.** Immucor and One Lambda/ThermoFisher assays have a similar, albeit nonidentical, ability to detect anti-HLA Ab. Although the correlation between the assays was present, significant variances exist, some of which can be explained by a dilution-sensitive "prozone" effect.

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The detection of anti-HLA antibodies (Ab) is essential for the evaluation and immunomonitoring of solid organ transplant recipients. The presence of anti-HLA Ab in sensitized transplant candidates reduces the pool of suitable donors and increases wait time. De novo anti-HLA Ab-detected posttransplant are associated with an increased risk of cellular and Ab-mediated rejection (AMR)[1-5] and death.[6-8] Historically, anti-HLA Ab were detected using complement-dependent cytotoxicity (CDC) assays.[9] This technique has now been complemented or in some instances supplanted by solid phase assays using beads coated with HLA antigens and a Luminex apparatus. Not only does the Luminex-based platform allow for the determination of the anti-HLA Ab specificity but it also helps evaluate their binding strength

through the measurement of mean fluorescence intensity (MFI).[10] This assay is more sensitive than the traditional CDC method.[11] At the same time, substantial variations have been observed in MFI measurements using different kits or between different laboratories, limiting the interpretation of the test.[12] Additionally, there have been reports of discordant results between manufacturers.[13] In this study, we sought to compare the performance of One Lambda/ThermoFisher and Immucor single-antigen assays for the detection of anti-HLA Ab.

MATERIALS AND METHODS

Patient Specimens and Clinical Information

This study used 125 serum specimens collected before (n = 17) or after (n = 108) heart (n = 120) or lung (n = 5) transplantation and archived in our HLA laboratory repository. To ensure diversity, specimens were randomly selected to include sera with different reactivity profiles: (1) negative by CDC, negative by OneLambda/ThermoFisher, (2) negative by CDC, positive by OneLambda/ThermoFisher for HLA class I Ab, (3) negative by CDC, positive by OneLambda/ThermoFisher for HLA class II Ab, (4) negative by CDC, positive by OneLambda/ThermoFisher for HLA class I and class II Ab, (5) positive by CDC, positive by OneLambda/ThermoFisher for HLA class I and class II Ab. A positive Ab was defined by a background-adjusted MFI cutoff of 1000 or greater. Serum samples were tested by CDC and OneLambda/ThermoFisher kits as part of their routine clinical care at our center (protocol below). Archived frozen aliquots of the same sera were sent to Immucor Inc. (Stamford, CT) for blinded testing after deidentification. This study was performed under Columbia University Medical Center IRB-AAAO3904.

One Lambda/ThermoFisher Protocol

Pretransplant and posttransplant sera were tested for class I and class II anti-HLA Ab using commercial Single Antigen Flow Beads on the Luminex platform (LABScreen single antigen, One Lambda Inc., Canoga Park, CA). Our laboratory performed the test according to the manufacturer's protocol. LABScreen products use color-coded microbeads coated with purified class I or class II HLA antigens. The neat (undiluted) serum was first incubated with LABScreen beads for 30 minutes; wash buffer was then added to the bead/serum (washed 3 times) and then diluted antihuman IgG phycoerytrin (PE) conjugate was added. Anti-HLA Abs present in the test serum were bound to the antigens on the beads and then were labeled with R-PE–conjugated Goat antihuman IgG. The LABScan 100 flow analyzer was used to detect the fluorescent emission of PE from each bead. The reaction pattern of the test serum was compared to the lot-specific worksheet defining the antigen array and assigned the HLA specificity. Results were interpreted of using HLA FUSION software (One Lambda) and expressed as MFI. A positive result was defined when the background-adjusted MFI was ≥1000.

Immucor Protocol

Like the One Lambda/ThermoFisher kit, the Immucor LIFECODES LSA Single Antigen kit uses recombinant HLA molecules for all HLA-A, HLA-B and HLA-Cw and 90 HLA-DRB, HLA-DQB and HLA-DPB. Serum samples were analyzed for the presence of class I and class II anti-HLA Ab using LIFECODES LSA Single Antigen Antibody detection kits (Immucor, Norcross, GA) following the manufacturer's recommendations. Briefly 10 μL of serum was centrifuged to remove debris and then incubated with 40 μL beads for 30 minutes. After a wash, the diluted antihuman IgG PE conjugate was added to the beads. After a final 30-minute incubation, wash buffer was added to the wells, the plate was placed in the Luminex instrument, and data were collected for analysis. Data were analyzed using the MATCH IT! Antibody software, which uses the analysis method as described in the LIFECODES LSA product insert. The background values used to calculate the background-adjusted MFI values were provided by the manufacturer and are derived from the average MFI of a panel of negative sera. A positive result was defined when the background-adjusted MFI was ≥1000.

Statistical Analysis

Background-adjusted MFI values were used from both platforms for comparison. Bivariate correlations (2-sided tests) were performed using Pearson's correlation coefficient and linear regression. Regression diagnostics were performed to identify outliers and influential points using leverage, Studentized residuals, Cook's D ($\frac{4}{n}$ cutoff), and DFBETA ($\frac{2}{\sqrt{n}}$ cutoff). MFI variation between assays by individual bead was expressed via box plots, displaying the mean, median, interquartile range, minimum, and maximum values. Lastly, Bland-Altman plots were used to compare MFI values for paired antigens between the 2 manufacturers, with mean bias and 95% limits of agreement determined. A 2-tailed P value of less than 0.05 was considered significant. Analyses were performed using SAS version 9.4 (SAS Institute, Inc, Cary, NC).

RESULTS

All sera combined, 1284 HLA class I Ab were detected using the OneLambda/ThermoFisher kit and 1010 were detected using the Immucor kit with an MFI cutoff value of 1000; 916 were detected by both (Figure 1A). When the MFI threshold was increased to 4000, there was greater agreement between manufacturers (Figure 1A). For HLA class II, there were reactions to 766 antigens using the One Lambda/ThermoFisher kit and 541 antigens using the Immucor kit; 458 were detected by both kits. The agreement between the 2 platforms increased when the positive MFI cutoff was increased to 4000 (Figure 1B).

All MFI values obtained using the 2 platforms were then compared quantitatively. For paired HLA class I values ie, positive using both methods, there was a weak positive correlation between the 2 manufacturers ($r = 0.3$, $r^2 = 0.09$, $P < 0.0001$, Figure 2A). Visual inspection of the scatter plot and regression diagnostic tests (**Figure S1, SDC,** http://links.lww.com/TP/B482) identified a cluster of strongly discordant paired values: 57 with high (>16 000) Immucor values and low One Lambda values (<5000), 3 with high (>16 000) Immucor values and intermediate (5000-6000) One Lambda values, and 2 with high (>16 000) One Lambda values and low Immucor values (<5000) (**Table S1, SDC,** http://links.lww.com/TP/B482). Correlation and regression analysis was then repeated after removal of these highly discordant values. Excluding these outliers increased the degree of correlation ($r = 0.59$, $r^2 = 0.35$, $P < 0.0001$, Figure 2B). For HLA class II, we observed a strong positive correlation between values obtained using the 2 separate kits ($r = 0.707$,
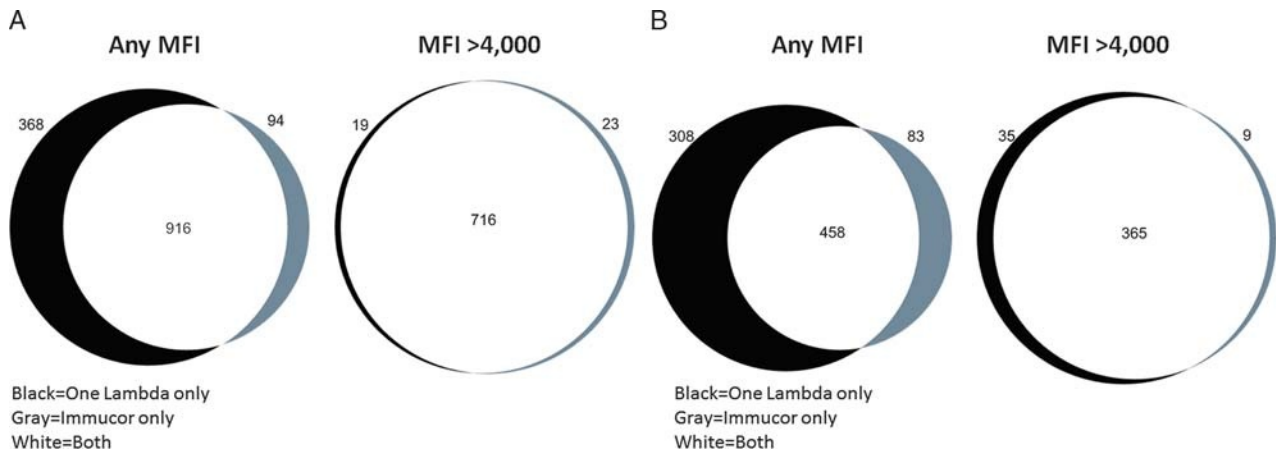
**FIGURE 1.** Comparison of class I (A) and class II (B) anti-HLA Ab detected by different assays.

$r^2 = 0.5$, $P < 0.0001$, Figure 3). Again, several outliers could be distinguished but the amount of MFI discordance was less pronounced (**Table S1, SDC,** http://links.lww.com/TP/B482). The correlation increased after omitting these outliers ($r = 0.82$, $r^2 = 0.67$, $P < 0.0001$). Logarithmic transformation did not markedly improve either class I ($r = 0.47$, $r^2 = 0.22$, $P < 0.0001$) or class II correlation ($r = 0.75$, $r^2 = 0.56$, $P < 0.0001$).

This population of outliers was further investigated because there was suspicion that assay interference was playing a role. Sera from 11 patients, which accounted for over 80% of the outliers, were retested using the One Lambda kit following 1:4 dilution with PBS (phosphate-buffered saline). On initial testing for these patients, there was no correlation among the class I and a very weak correlation for class II (Figures 4A and C). After dilution, there was a clear improvement in the correlation between different vendors (Figures 4B and D).

Bland-Altman plots were generated to quantify bias (mean difference) and the range (95% confidence interval [CI]) of MFI values was obtained by the 2 methods. For class I values, no significant MFI bias was observed overall, even though there was a wide range (95% CI, –13 203 to 13 193; Figure 5A). The previously identified cluster of outlier values was again evident and appeared to bias the overall mean. A repeat analysis excluding these values revealed a bias with

One Lambda/ThermoFisher MFI values being significantly higher (Mean Difference 1291) than values obtained by Immucor with a narrower range (95% CI, –7550 to 10133). Results for HLA class II followed a similar pattern (Figure 5B). When stratified by mean MFI the variance between assays increased as the mean MFI increased (**Figure S2A and S2B, SDC,** http://links.lww.com/TP/B482), with a systematic bias toward greater MFI values generated using the One Lambda kit. We then evaluated differences in MFI values obtained for individual HLA alleles using the 2 platforms (**Figure S3A and S3B, SDC,** http://links.lww.com/TP/B482). Despite the kits differing in the specific HLA antigens screened (**Table S2, SDC,** http://links.lww.com/TP/B482), no single HLA serological type appeared to be recognized preferentially by 1 kit more than the other (**Tables S3 and S4, SDC,** http://links.lww.com/TP/B482).

Aside from paired values, several individual HLA Ab were detected by only 1 platform in several serum specimens. Immucor detected 94 class I Ab that were not detected by One Lambda/ThermoFisher; 71 had an MFI of less than 4000 (**Figure S4A, Table S3, SDC,** http://links.lww.com/TP/B482). Conversely, One Lambda detected 368 class I Ab, 349 of which had an MFI value less than 4000. There were similar findings for HLA class II, with 308 Ab detected by One Lambda alone and 83 detected by Immucor alone (**Figure S4B, Table S4,**
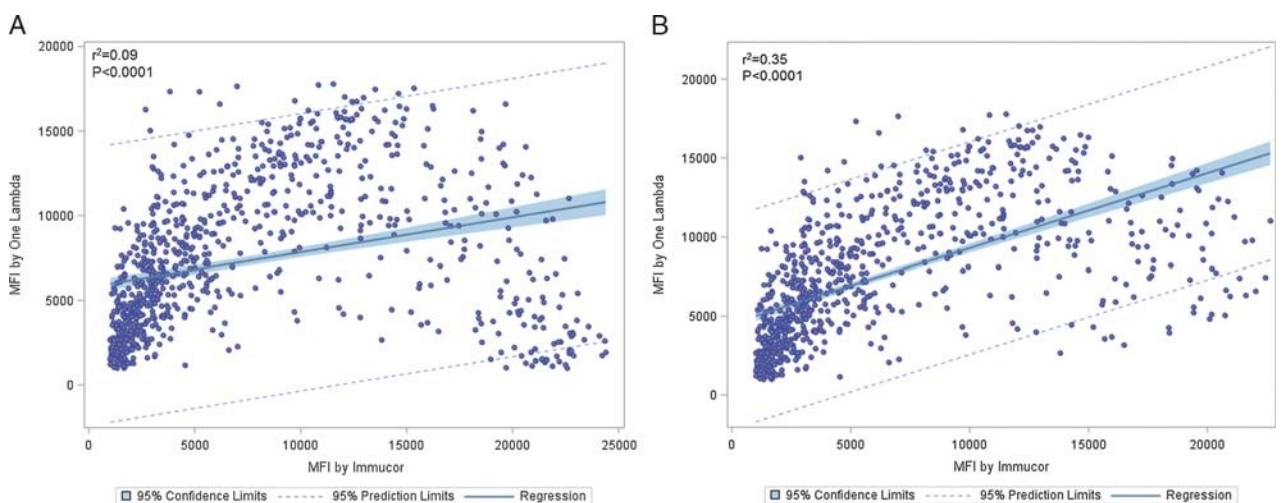
FIGURE 2. Correlation for (A) All class I Ab and (B) class I Ab excluding outliers.
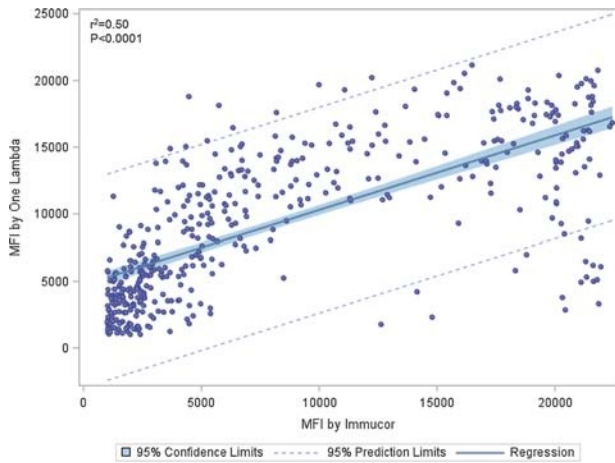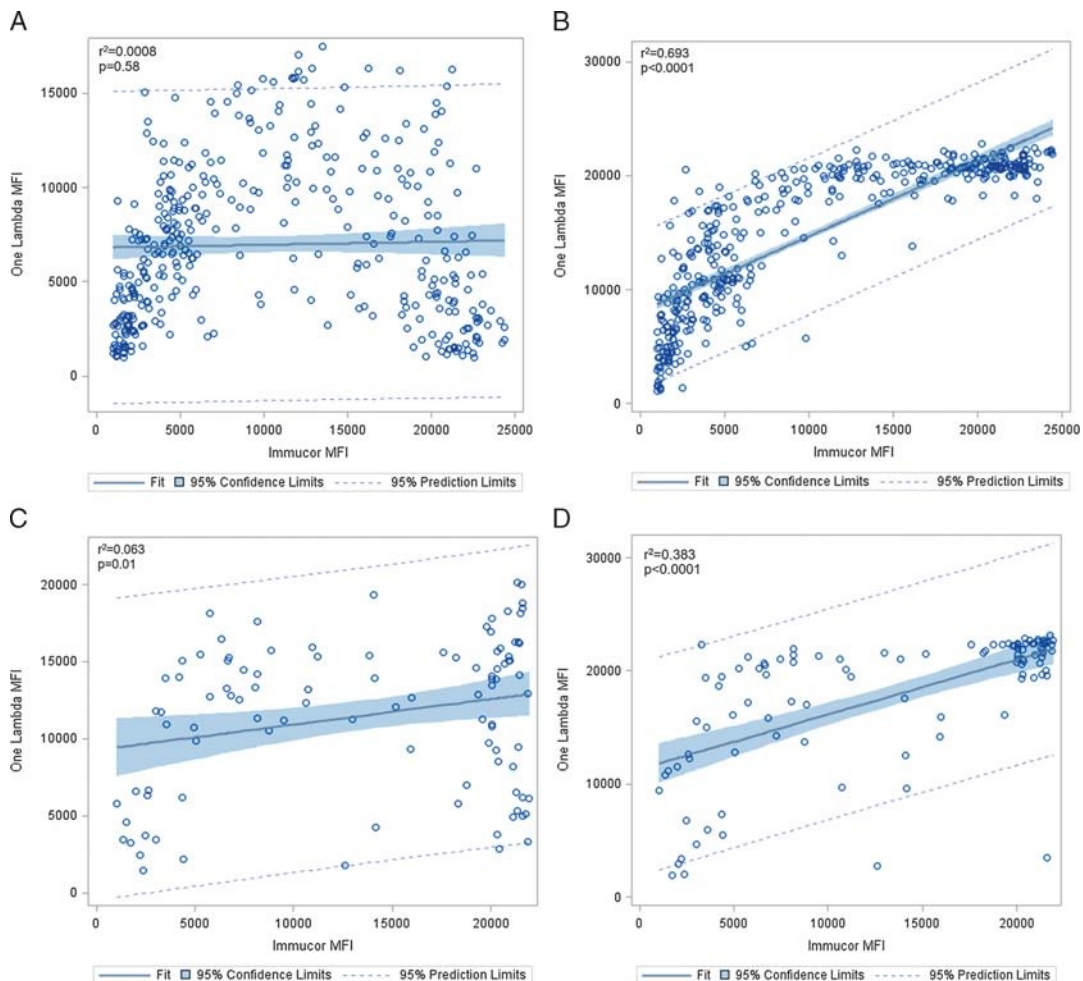
FIGURE 3. Correlation for all class II Ab.

SDC, http://links.lww.com/TP/B482). Only 35 for One Lambda and 9 for Immucor Ab had an MFI greater than 4000.

Among all Abs detected only using the Immucor kit, 9 corresponded to donor-specific antibodies (DSA) in heart transplant recipients (Table 1). Three were detected in pretransplant samples, 2 of which had an MFI value below

5000 that at our institution would not have necessitated a prospective crossmatch (Cw17 [1035] and DP2 [3619] and DQ8 [1078]). However, the Immucor kit also detected anti-DP4 Ab not seen with the One Lambda kit in 1 patient with an MFI of 5054, which would have required a prospective crossmatch. None of the 3 patients experienced AMR during the brief posttransplant follow-up period (6-10 months). DSA were also detected in posttransplant serum samples in 6 patients: 4 with low level class I Ab (MFI, 1017-1486), 3 with low level class II Ab (MFI, 1132-2199), and 1 with moderate class II Ab (MFI 6644). None of these 6 patients experienced AMR in the period immediately after the blood collection that tested positive for DSA using the Immucor kit.

DISCUSSION

Detection and quantification of anti-HLA Ab are an integral component of solid organ transplant recipient immunomonitoring. This study sought to compare the performance of the 2 main Luminex-based assays commercialized by Immucor and One Lambda/ThermoFisher. The study resulted in 4 principal findings: (1) both assays were grossly comparable in their ability to detect the clear majority of anti-HLA class I and class II Ab. A positive correlation was

FIGURE 4. Correlation between One Lambda and Immucor Assays for class I before (A) and after (B) dilution for and class II before (C) and after (D) dilution.
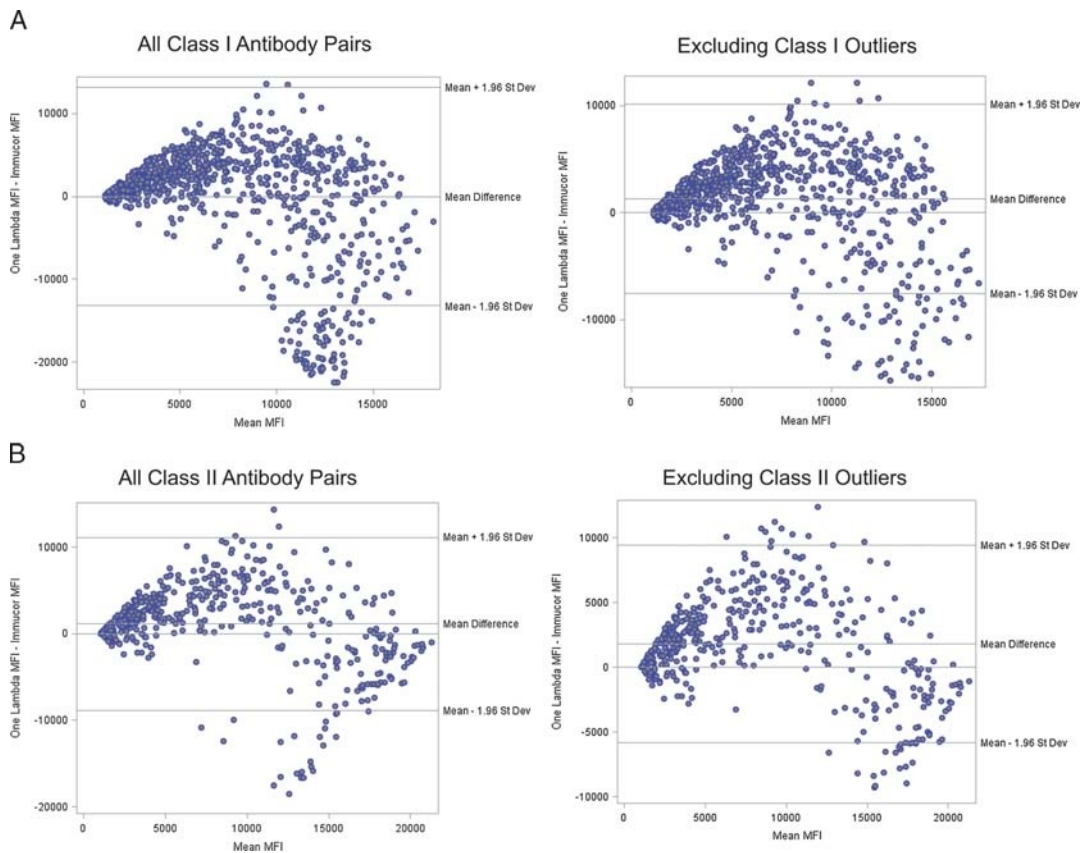
FIGURE 5. Bland-Altman plot comparing One Lambda and Immucor class I Ab (A) and class II Ab (B).

observed between MFI values obtained from both assays. (2) There were, however, significant differences between the 2 platforms as each assay detected moderate to high titer Ab that the other did not. (3) Ab detected by only 1 assay did not target any specific HLA allele. Rather, different results were obtained for all Ab in specific samples. (4) For these specific serum samples, dilution before testing with the One Lambda/ThermoFisher beads increased the convergence between the 2 assay results. These findings are discussed below.

The general qualitative correlation between the 2 assays was balanced by a weaker quantitative correlation between

MFI. Class I Ab displayed a weak positive correlation when all Ab paired values were considered, and the strength improved when outliers were excluded. Similarly, there was a strong positive relationship between the 2 assays for the class II Ab. Despite the monotonic relationship and the statistical correlation, the variation in Ab intensity between the 2 assays was notable. The mean difference overall was small, but when stratified by mean MFI, the mean differences ranged from 1274 to 4659 for class I and 1321 to 3239 for class II with broad 95% confidence intervals. The difference between the manufacturers is not entirely surprising. Previous work revealed that MFI values differ between kits from the

TABLE 1.

Clinical outcomes

Patient	DSA	Serum timing	Immucor MFI	Outcomes	Follow-up
1	DP4	Pretxp	5054	No rejection, no DSA against this antigen	9 mo
2	Cw17	Pretxp	1035	Immediate 2R/3A rejection, no AMR, no DSA against this antigen	10 mo
3	Cw2, Cw7	Posttxp	1017 and 1290	No rejection, no DSA against this antigen	6 y
4	DR4	Posttxp	1668	No rejection, no DSA against this antigen	14 mo
5	DP2	Posttxp	6644	No rejection, no DSA against this antigen	16 mo
6	DR13	Pretxp	2199	No rejection, no DSA against this antigen	3 y
7	DP2, DQ8	Pretxp	3619 and 1078	No AMR, (had 1R/1B), no postop DP2 or DQ8 (Subsequently developed DP2 pretxp but MFI < 5000 and C1q negative)	10 mo
8	Cw4, Cw5	Posttxp	1486 and 1288	No rejection, no DSA against this antigen	30 mo
9	DQ2, DQ8	Posttxp	1132	No rejection, no DSA against this antigen	2 y

same manufacturer by 310 to 1500 and averaged MFI (measured for all Ab) can also vary as much as 700.[12] However, the magnitude of the variation between values obtained by the 2 assays in our study was unexpected. Although this finding needs independent confirmation, it limits the comparison of measurements between the 2 platforms. It is unclear which set of values has more clinical relevance.

Despite being overall consistent with one another, results obtained using the 2 assays also revealed several discrepant measurements when considering all anti-HLA Ab with an MFI above 1000. Most differences were observed for low MFI values (MFI < 4000), with the One Lambda assay having greater reactivity to anti-HLA Ab in that range. Many of these anti-HLA Ab recognized by the One Lambda kit only (4000 > One Lambda MFI > 1000) were in fact also detected by the Immucor kit, but below the threshold for this study (MFI < 1000). It appeared therefore that much of the discrepancy between the kits for these low MFI anti-HLA Ab was due to the different MFI levels measured by each kit. Although anti-HLA Ab with an MFI above 5000 are generally considered meaningful pretransplant using the One Lambda kit, Ab generating lower MFI values have less certain clinical implications.[14]

A cluster of outlier values was also identified using regression diagnostics for both anti–class I and class II Ab. We first reasoned that Ab to some specific HLA were detected with greater efficacy by 1 or the other assay. However, this did not appear to be the case. Further analysis revealed that these differences were primarily observed for a few individual serum samples. Nearly 75% of all anticlass I and class II Ab outliers were from 3 and 2 patients, respectively. Moreover 100% of anticlass I and 95.4% of anticlass II outliers were obtained by testing sera from highly sensitized patients. In addition to the outliers, there were several strong (MFI > 10 000) anticlass I and anticlass II Ab that were only detected by 1 assay (**Figures S4 and S5, SDC**, http://links.lww.com/TP/B482). Both assays were performed per the manufacturer's recommendations; however, the Immucor protocol includes dilution (sera are diluted 5-fold in the Immucor LIFECODES assay) of the sample. Dilution is known to reduce assay interference due to the "prozone" effect[15] (resulting from the presence of high titer HLA Ab), complement interference, and HLA-specific IgM Ab. Diluting the sera restored the MFI value in conventional IgG testing providing a plausible explanation for the high titer MFI anti-HLA Ab detected by Immucor but not by the One Lambda kit in some samples. Retesting of samples that included outliers on the One Lambda platform after dilution generated results consistent with the Immucor measurements, strongly suggesting that the original discrepancies resulted from the "prozone" effect.[16] Retesting diluted serum is costly and increases the workload of individual HLA laboratories and alternatives such as addition of ethylenediaminetetraacetic acid[17,18] or dithiothreitol[16] to serum before testing have been proposed. The reason why some One Lambda MFI values exceeded Immucor MFI values for the same samples is still unknown.

This study has several limitations. First among them is the single center nature of the study. All One Lambda values were generated in our histocompatibility lab as part of routine clinical care, over a 2-year period using different batches of kits. In contrast, Immucor measurements were generated blindly by Immucor in 2 batches. Interkit variations may account for some of the differences we observed. Lastly, this study was not designed to follow clinical outcomes and is therefore predominantly descriptive. We could not determine if the differences between the assays had any clinical implication.

In conclusion, Immucor and One Lambda assays display a comparable ability to detect medium or greater titer (MFI ≥ 4000) anticlass I and anticlass II Ab. However, significant differences were observed between the 2 assay results, mostly associated with specific serum specimens. Dilution of sera (especially of highly sensitized patients) resulted in greater agreement between the 2 assays, reducing interference likely related to the "prozone" effect seen in some individual sera from highly sensitized patients.

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