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Background. Ethylenediaminetetraacetic acid (EDTA) pretreatment has been shown to overcome complement interference in the single-antigen bead (SAB) assay. However, a quantitative evaluation of its impact on the assay for preemptive application to diverse clinical samples is still lacking. **Methods.** Serum samples from 95 renal transplant candidates were tested with and without EDTA-pretreatment in parallel. Changes in mean fluorescence intensity (MFI) values were analyzed to determine the impact of EDTA-pretreatment and the characteristics of complement interference. **Results.** MFI values from EDTA-treated and untreated sera showed good correlations (r = 0.99) and were linear after excluding outliers (slopes, 1; intercepts, −63.7 and −24.2 for class I and II, respectively). Using an assay cutoff of 2000 MFI, positive/negative assignments were concordant for 99% of the 9215 class I beads and 9025 class II beads tested. As defined by an MFI increment above 4000 after EDTA pretreatment, complement interference affected 172 class I beads in 12 samples (12.6%) and 60 class II beads in 7 samples (7.4%), and the findings were supported in 83% and 86% of these samples by dilution studies. In a case study, EDTA pretreatment prevented falsely low MFI values and facilitated the interpretation of titration curves. Finally, EDTA pretreatment reduced the coefficient of variance (CV) by 2.1% and 2.4% for class I and II beads respectively (*P* < 0.0001). **Conclusions.** It is safe to preemptively treat all clinical samples with EDTA before SAB assay to prevent false negative results or falsely low MFI values. EDTA pretreatment has the added benefit of improved assay precision.

Single-antigen bead (SAB) assays allow detection of antibodies to specific human leukocyte antigens (HLA), which enable virtual crossmatches and efficient organ allocation to sensitized patients.[1-3] In the posttransplant setting, detection of donor-specific antibodies against HLA (DSAs) has become an important criterion for diagnosing antibody-mediated rejection (AMR)[4,5] and a prognostic factor for long-term graft outcomes.[6] However, limitations of SAB assays have been recognized,[1,7] such as substantial inter-laboratory and inter-run variations,[8,9] the inconsistent correlation between the mean fluorescence intensity (MFI) and the clinical significance of an antibody,[7,10] and spurious results due to various interfering substances.[11,12]

Complement interference of the SAB assay has captured the attention of many HLA laboratories and clinicians in the transplant community recently.[12] This phenomenon mimics a 'prozone effect' where antibodies undetectable in neat serum samples are highly positive when tested after dilution. Elegant studies have elucidated the underlying mechanism, which involves the activation of complement factors on the beads blocking the access of reporter antibodies.[12-14] Although few would dispute this mechanism, the arbitrary definition of complement interference varies among studies (Table 1).[12-16] There are also other known causes of interference

TABLE 1.

Definitions of complement interference or 'prozone effect' in representative studies

Study	Definition of complement interference	Subjects included in the study
Schnaidt, 2013	A 2-fold increase of the MFI after 1:10 dilution (or other serum pretreatment)	A few highly sensitized kidney and stem-cell transplant candidates
Schwaiger, 2014	A greater than 100% increase in IgG MFI after 1:10 dilution	12 broadly sensitized kidney transplant candidates
Visentin, 2014	>1 bead with > 2 fold increase in MFI to > 10 000 after EDTA pretreatment compared to results with neat serum	1 lung, 2 liver, 1 heart recipients who are HLA-sensitized
Tambur, 2015	Neat MFI value is lower than the MFI value in the subsequent dilution(s)	55 transplant patients or candidates with strong antibodies (MFI > 10 000), or with clinical indication for a titration study
Anani, 2016	The appearance (or loss) of a new antibody and/or a change of >2000 MFI after EDTA treatment	10 pretransplant and 48 posttransplant specimens with suspected prozone effect (lung 22, heart 10, kidney 21, heart/lung 2, pancreas 1, small bowel 1)

in the single antigen assays, including true prozone due to high antibody levels. While many studies focused on highly sensitized patients and beads with the strongest complement interference for analysis, a quantitative evaluation of the global impact of complement interference on individual beads is lacking especially in nonhighly sensitized patients.

The clinical importance of complement interference has been demonstrated in case reports where false negative results from the SAB assay or flow cytometric crossmatch[17-19] may have adversely affected patient care. Although multiple methods, including titration, EDTA or dithiothreitol (DTT) pretreatment, and heat inactivation, have been shown to abolish the complement interference,[12-16,19,20] it is not always possible to predict which sample will be affected by complement interference. It is impractical to use all methods routinely in a histocompatibility laboratory but is reasonable to select 1 method and apply the method preemptively for all samples tested by the SAB assay. To our knowledge, several laboratories in the United States have already implemented EDTA pretreatment for all SAB testing. However, it remains unclear how to validate such a significant modification of an FDA approved assay. Among the biggest challenges are the potential dilution effect of the EDTA pretreatment, the lack of a consensus definition or gold standard for complement interference, and the quantitative analysis of complicated data sets from the validation.

In this cross-sectional study, we evaluated the impact of preemptive EDTA pretreatment on the SAB assay in kidney transplant candidates with a broad spectrum of sensitization. We also investigated the landscape of complement interference as revealed by EDTA pretreatment, and report additional benefits of EDTA pretreatment that heretofore were not fully recognized.

MATERIALS AND METHODS

Serum Samples

Serum specimens from 95 kidney transplant candidates at our center were included in this study (20 were consecutive specimens beginning August 2016 and 75 were drawn from historical specimens based on computer-generated random numbers). All sera were frozen before testing. To evaluate the effect of EDTA pretreatment in combination with titration, serial samples from a heart transplant recipient with AMR treated with therapeutic plasma exchange (TPE) were tested. To evaluate the inter-run variation of the assay, a positive control serum pooled from multiple highly sensitized

patients was included. Institutional review board approval was exempted for this quality improvement project.

Testing Methods and Algorithm

Each serum sample was tested with and without EDTA pretreatment in parallel using the LABScreen SAB kits (LS1A04 lot 009 for class I and LS2A01 lot 011 for class II; One Lambda, Canoga Park, CA) on the Luminex 200 instrument (Luminex Corporation, Austin, TX). The product manual was followed except that 25 μL of beads was used per test (per long-standing institutional protocol). Serum samples were pretreated with EDTA by mixing 5 μL 6% EDTA solution (6 grams of dipotassium EDTA in 100 mL water, pH = 8.0; Sigma-Aldrich, St Louis, MO) with 95 μL serum before the SAB assay.[12] Sera positive for complement interference (1 or more beads with MFI increment > 4000 MFI after EDTA pretreatment) were subsequently tested at 1:5 and 1:25 dilutions in 5% BSA in Hank's balanced salt solution (HBSS). For the heart transplant patient, 3 serum samples from before initiation of TPE and after the fifth and 8th procedures were tested at neat and after 3 serial 1:5 dilutions up to 1:125 with and without EDTA pretreatment.

Definitions of Complement Interference and Cutoff Values

We examined complement interference based on 2 different definitions and a range of cutoff values that took into consideration criteria reported previously[12-16] (Table 1). First, a fixed increment in MFI value after EDTA pretreatment was used as a cutoff to identify beads affected by complement interference.[15] For sensitivity analyses, we varied the MFI increment cutoff between 400 and 4000 to demonstrate a gradient of sensitivity. The lower bound of a 400 MFI increment approximates the inter-run variance around the assay cutoff of 2000 in our laboratory,[21] while the higher bound was an arbitrary choice (2 times the 2000 MFI increment used in Ref 15). Second, a percent increase in MFI value after EDTA pretreatment compared to neat serum was used as another cutoff to identify beads affected by complement interference.[12-14] A range of cutoff values from 20% to 200% was included for sensitivity analysis.

Data Analysis and Statistics

All 95 samples passed the quality control (MFI of negative control beads < 1500, and MFI of positive control beads > 5000) and were included in the final analysis. Background-normalized MFI values for all beads were downloaded from Fusion V3.0 (One Lambda, Canoga Park, CA) and indexed per product catalog, patient study code, bead specificity and testing

method using the Python programming language V2.7.[22] The impact of EDTA pretreatment and the characteristics of complement interference were visualized using the package Matplotlib v1.5.3.[23] Linear regression and paired t test were implemented using the Stats package of SciPy v0.18.0.[24] P values below 0.05 were considered statistically significant. cPRA was calculated for samples with discordant results based on the antigen specificities with MFI values above 2000 using a validated online tool.[25] DP specific antigens were not entered for cPRA calculation, which is a known limitation of the OPTN cPRA calculator.[26] Paired cPRA values from neat and EDTA-treated sera were then plotted for class I and class II separately using Microsoft Excel (2016 MSO, Version 16.0.7127.1026).

RESULTS

Correlation of MFI Values from Neat Sera Versus EDTA-Treated Sera

When neat sera and EDTA-treated sera were tested in parallel, the 2 methods were comparable with most of the MFI data points distributed along the line of agreement (Figure 1A, B). MFI values were elevated substantially for a small number of beads after EDTA pretreatment, a pattern consistent with complement interference reported in the literature.[12,13] The distribution of all data points appeared nonlinear due to these outliers, and linear regression was only appropriate after the attempt to exclude the outliers arbitrarily defined as beads with MFI increment above 4000 after EDTA pretreatment (see definitions below). MFI values from the EDTA-treated and -untreated sera indeed showed good correlations (r = 0.99, $P < 0.001$) and were linear with slopes of 1 and small negative intercepts, –64 and –28 for class I and II respectively. These data suggest that EDTA pretreatment does not significantly impact the SAB assay other than reducing the presumed complement interference.

Discordant Results from Neat Versus EDTA-treated Sera

Compared to results from neat sera using a cutoff MFI of 2000, EDTA pretreatment led to discordant results for 86 class I beads in 29 samples (1% of all class I beads). The results on 70 of these beads (81%) changed from positive to negative, and 16 beads (19%) negative to positive (Figure 2A). A total of 47 specificities were affected, and the counts of discordant events per bead varied between 1 and 5. The median change in cPRA after EDTA treatment for these samples was –1% (range, –41% to 3%; Figure 2C).

Results after EDTA pretreatment were discordant for 92 class II beads in 26 samples (1% of all class II beads), with 58 beads (63%) changing from positive to negative and 34 beads (37%) from negative to positive (Figure 2B). A total of 35 specificities were affected, and the counts of discordant events per bead varied between 1 and 4. The median change in cPRA after EDTA treatment for these samples was 0% (range, –30% to 60%; Figure 2C).

Among discordant beads that changed from positive to negative with EDTA pretreatment, 97% of the beads had MFI values below 3000 with neat sera. Among discordant beads that change from negative to positive, 26% and 14% of the beads had increased MFI values to above 3000 and 20 000 respectively after EDTA pretreatment. With the assay cutoff values increasing from 1000 to 5000, the number of discordant beads trended down, and the majority of the discordant beads changed from weakly positive to negative (insets of Figure 2A and 2B).

Landscape of Complement Interference Revealed by EDTA Pretreatment

To characterize the frequency of complement interference in the study population, we started with a sensitive cutoff of 400 MFI increment after EDTA pretreatment. Complement interference defined by this criterion affected 343 class I beads (3.7%) in 21 samples (22%) and 242 class II beads (2.7%) in 22 samples (23.2%). By increasing the cutoff to 4000, the frequency decreased to 172 class I beads (1.9%) in 12 samples (12.6%) and 60 class II beads (0.7%) in 7 samples (7.4%). By plotting the numbers of affected beads per sample and over a range of cutoff values (Figure 3A and B), we not only visualized individual affected samples as colored lines, but
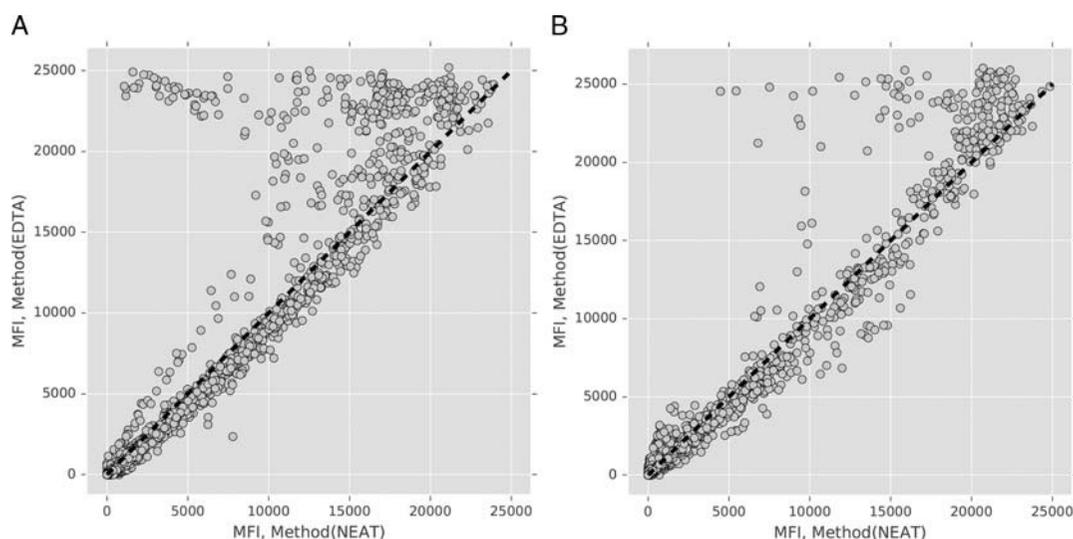
FIGURE 1. Comparison of MFI values from neat serum and serum treated with EDTA. MFI values of class I (A) and class II (B) beads from neat and EDTA-treated serum samples are visualized using scatter plots. Dashed lines along Y = X serve as the reference. Ninty-five evaluable samples are included for this analysis; class I beads, N = 9215; class II beads, N = 9025.
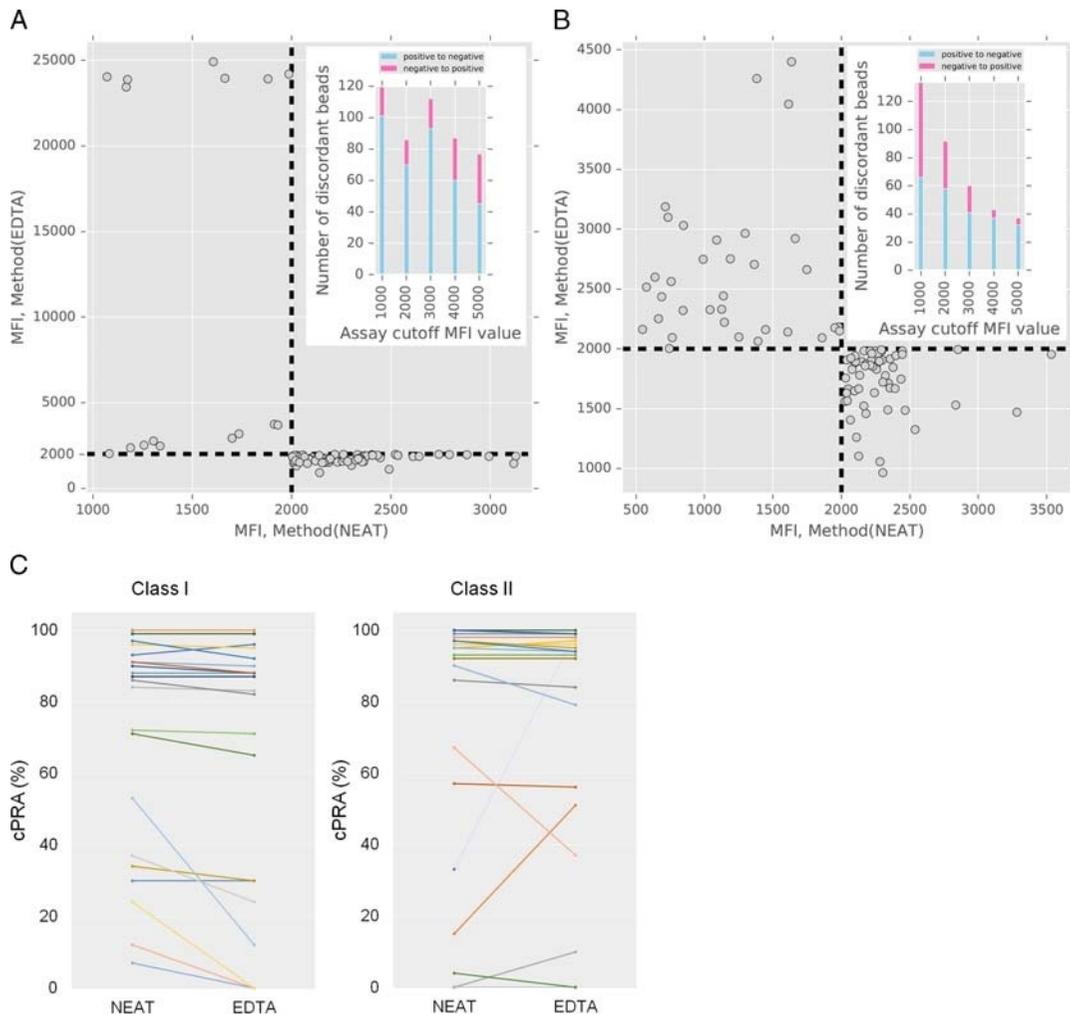
FIGURE 2. Discordant results from SAB assay with neat or EDTA-treated sera. MFI values from discordant beads with neat sera (x-axis) and EDTA-treated sera (y-axis) were plotted in (A) and (B) for class I and II beads respectively. Insets demonstrate the number of discordant beads with different cutoff MFI values for the SAB assay. The stacked bars show 2 categories of discordant results, beads changing from positive to negative after EDTA pretreatment (blue) and beads from negative to positive after EDTA pretreatment (pink). C, The cPRA values from neat and EDTA-treated sera were plotted as pairs for samples with discordant results and for class I (left) and II beads (right) respectively.

also the heterogeneous compositions of complement interference within each sample that affected different numbers of beads to various degrees. The frequency of complement interference appeared to be lower when more stringent criteria were applied (Figure 3C and D). By counting beads with 100% increase in MFI after EDTA pretreatment, 53 class I beads (0.6%) in only 3 samples (3.2%) and 31 class II beads (0.3%) in only 6 samples (6.3%) were affected by complement interference.

Reexamination of Complement Interference by Dilution Study

To reexamine the complement interference by an alternative method, we performed dilution studies on samples with MFI increment above 4000 on 1 or more beads after EDTA pretreatment. Any increase in MFI after a 1:5 or 1:25 dilution was considered consistent with complement interference. Four patterns of serological reactivity were observed in these samples. Weaker complement interference can only find support at a 1:5 dilution but not 1:25 (Figure 4A and E). Stronger complement interference can find support at both dilutions,

sometimes with the MFI values higher at 1:25 than at 1:5 (Figure 4B and F). The weakest, presumed complement interference could not find support by dilution studies (Figure 4C and G). Samples with broad reactivity also frequently showed a mixture of the 3 patterns above (Figure 4D and H). Overall, complement interference could be supported by dilution studies in 83% and 86% of all samples with 1 or more class I and class II affected beads respectively.

Combining EDTA Pretreatment and Dilution Study

To evaluate how EDTA-pretreatment affects the upper range and linearity of the SAB assay, we performed a case study using samples from a patient undergoing TPE due to AMR mediated by high-MFI DSAs against DQ2 (Figure 5A-F). The 3 samples were drawn before the first TPE and immediately after the 5th and 8th procedures. The dilution study with and without EDTA pretreatment led to the following observations. First, 3 of the 5 DQ2 beads (DQB1*02 paired with DQA1*03, DQA1*04, and DQA1*05 respectively) showed sizable complement interference (Figure 5A-C) with neat serum but not EDTA-treated serum (Figure 5D-F). Second,
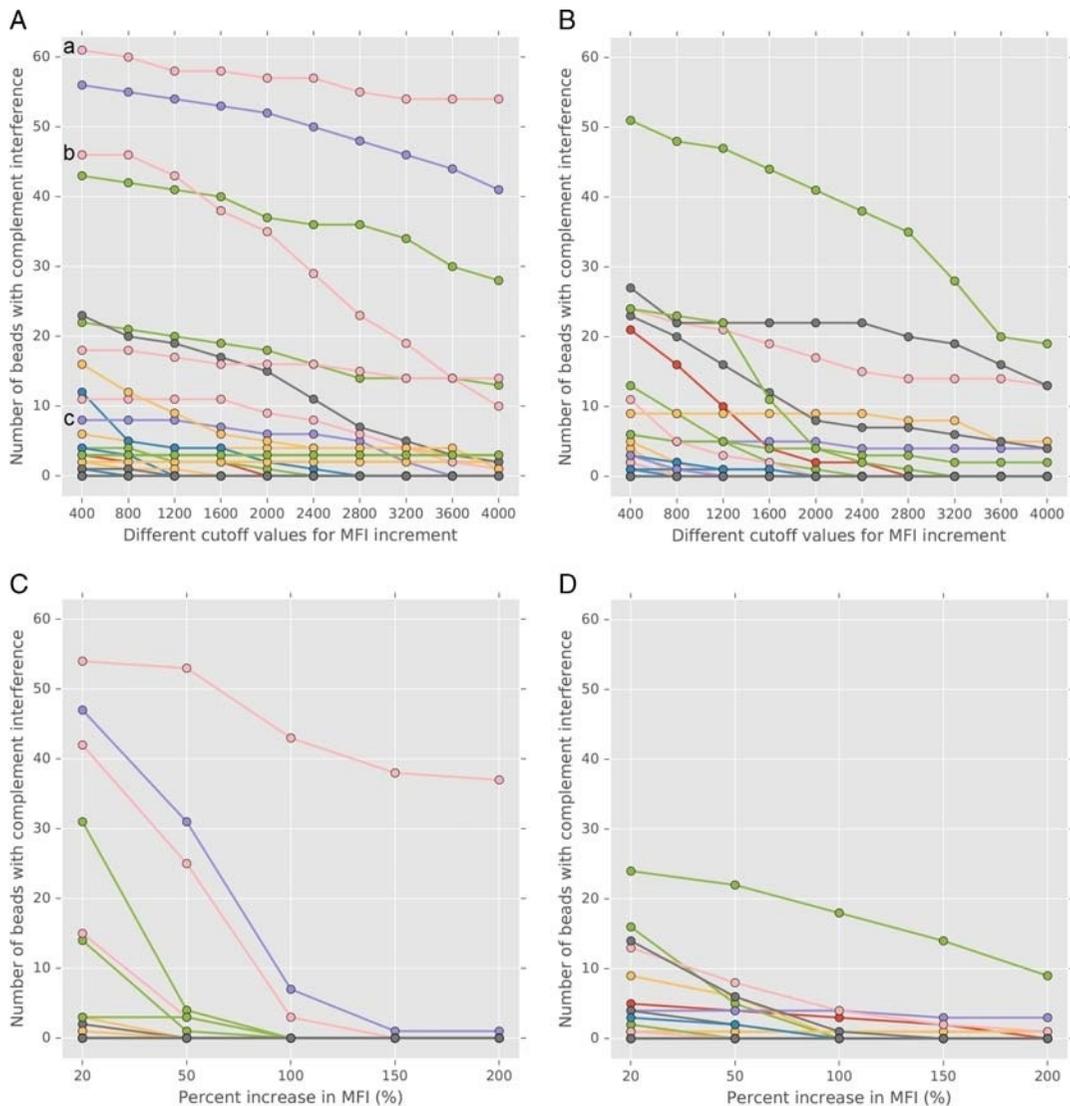
FIGURE 3. Effect of definitions and cutoff values on the observed frequency of complement interference. Each patient specimen is indicated by a colored line connecting dots that represent the numbers of beads affected by complement interference in that specimen (y-axis) as defined by a range of MFI increment cutoff values (x-axis). A and B, use absolute MFI increment after EDTA pretreatment, ranging from 400 to 4000, as the cutoff to define complement interference on each bead. Class I (A) and class II (B) beads are plotted separately and filtered on beads with MFI above 2000 using EDTA-treated sera. Three examples in (A) are described here: line "a" represents a sample with high levels of complement interference affecting a large number of beads (>50) almost uniformly; line "b" represents a sample with a broad spectrum of complement interference affecting beads to different degrees; line "c" represents a sample with low levels of complement interference affecting a small number of beads (<10). C and D, use percent increase in MFI with EDTA-treated serum over neat serum, ranging from 20% to 200%, as the cutoff to define complement interference on each bead. Class I (C) and class II (D) beads are plotted separately and filtered on beads with MFI above 2000 using EDTA-treated sera.

with EDTA-treated sera, all 5 DQ2 beads reached or approximated presumed peak MFI values without dilution, and a modest reduction in MFI was observed with further dilutions. In contrast for sera not pretreated with EDTA, 3 DQ2 beads reached peak MFI values only at dilutions of 1:25 (Figure 5B) or 1:125 (Figure 5A). Third, different DQ2 beads demonstrated different upper MFI limits around 25 000, 21 000, and 17 500, which may be related to different quantities of antigens coating individual bead. Finally, MFI values of the DSAs from EDTA-treated and -untreated sera were comparable at the titer of 125 but not at lower titers; the decreasing MFI values at the titer of 125 over the course of treatment indicated a modest benefit from TPE.

Precision of SAB Assay with EDTA-treated Sera

To test whether complement interference contributes to run-to-run variations of the SAB assay, we determined the CV by testing EDTA-treated and -untreated positive control serum in parallel over multiple independent runs (n = 24) performed by 5 technologists. The control specimen was pooled from highly sensitized patients, which allows the evaluation of CVs over a wide range of MFI values (Figure 6). Paired t test showed significantly lower CVs with EDTA pretreatment than without ($P < 0.0001$). The CVs were decreased by 2.1% (95%CI, 1.8-2.4%) and 2.4% (95%CI, 2.1-2.7%) on average for class I and II beads respectively (Figure 6A-B). When we looked at beads with MFI values between 1000 and 3000 (1000 MFI below and above our 2000 cutoff,
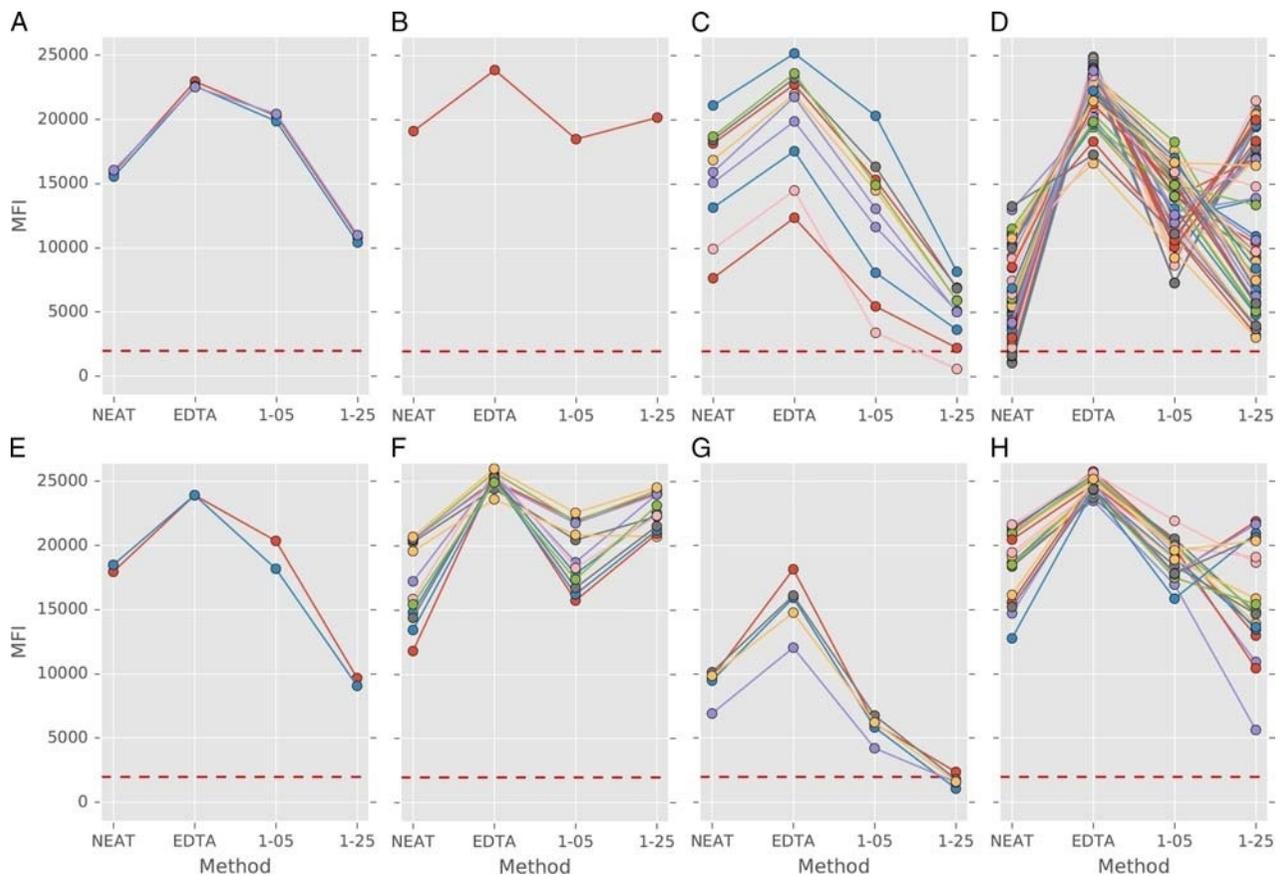
FIGURE 4. Reexamination of complement interference detected in EDTA-pretreated sera by dilution studies at 1:5 and 1:25. Four patterns are shown for class I (A-D) and class II (E-H) beads, respectively. Only beads with an MFI increment above 4000 after EDTA pretreatment were plotted, and each bead with MFI values from 4 different methods is represented by a colored line-dot plot. Pattern (A) and (E), complement interference is evident at the titer of 5 but not 25. Pattern (B) and (F), complement interference is more pronounced at the titer of 25 than 5. Pattern (C) and (G), complement interference demonstrated by EDTA pretreatment could not be supported by dilution studies at titers of 5 or 25. Pattern (D) and (H), a mixture of previous 3 patterns.

where the analytic precision is the most critical), EDTA pretreatment reduced CVs by 3.5% (95%CI, 1.5-5.6%) and 2.2% (95%CI, 1.6-2.8%) for class I and II beads, respectively.

DISCUSSION

Multiple studies have compared different methods for the abolishment of complement interference, showing comparable performances among these approaches,[12,16,19] and each method with its own strengths and limitations. We elected to validate preemptive EDTA pretreatment based on the following considerations. First, EDTA pretreatment of all samples undergoing SAB testing obviates the need for follow-up testing of selected samples and improves the turnaround time. Second, EDTA pretreatment is more cost-effective and less labor-intensive than dilution studies. Preemptive dilution studies may double or triple the reagent cost but benefit only a fraction of patients. Third, although the C1q assay can also overcome the complement interference,[13,16,18] it is a different assay with unique binding chemistry and perhaps decreased sensitivity for weaker antibodies. The C1q assay may not add much value if serum samples are tested by the SAB assay after dilution.[27]

It is critical to ensure that EDTA pretreatment does not significantly alter results from samples and beads not affected by complement interference. After excluding beads strongly

positive for complement interference (MFI increment above 4000), we observed a robust correlation between the results from EDTA-treated and untreated sera (Figure 1). Although the addition of EDTA solution was expected to dilute the serum samples to a small degree, this dilution effect was not detected. The negative intercepts of the regression imply a small constant negative bias of an unknown mechanism. The size of the bias appeared to be clinically insignificant and did not prompt us to adjust our assay cutoff value. The dilution effect remained undetectable when the regression line was forced through the origin. Moreover, only a small number of beads had discordant results between the 2 methods (Figure 2). For most samples with discordant results, 97% for class I and 81% for class II, there was either no change or a decrease in cPRA (Figure 2C) indicating a slightly increased chance of getting an offer. For rare samples with significant changes in cPRA, arbitrarily defined as more than 50% change in cPRA (n = 1 in this study), the impact on the affected patient could be substantial. Additional testing including titration and surrogate crossmatch may help prioritize the antigens to avoid. These results collectively demonstrated that preemptive EDTA pretreatment did not significantly alter the SAB assay other than removing the complement interference.

By including a range of MFI increments after EDTA pretreatment, our analysis was not limited by an arbitrary
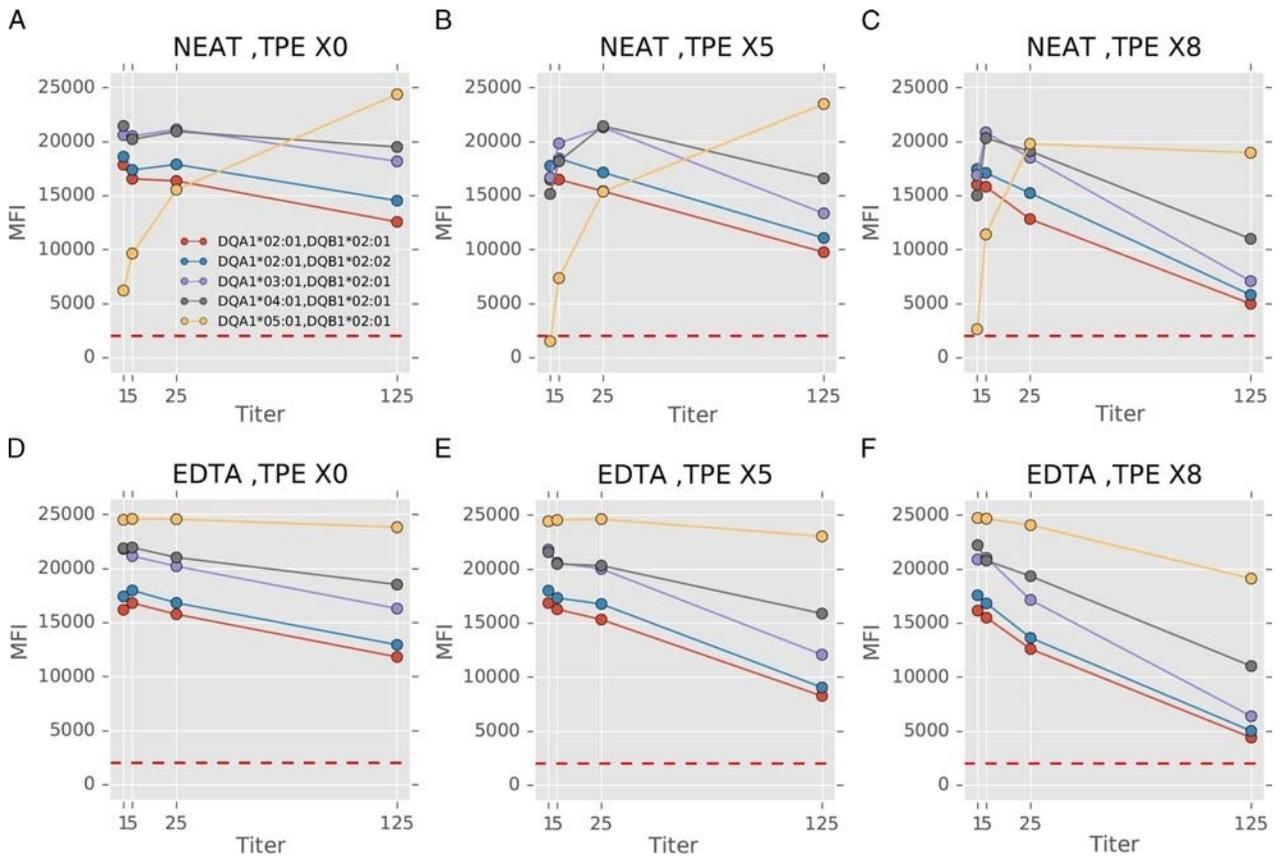
FIGURE 5. Upper range and linearity with EDTA pretreatment. Dilution studies with or without EDTA pretreatment were performed using 3 samples from a heart transplant patient with high-MFI DSAs against DQ2. The samples were obtained before TPE (A and D), after the fifth (B and E) procedure and the 8th procedure (C and F). The MFI values are plotted, and the broken red lines show the cutoff MFI value of 2000 for the SAB assay. Each color indicates a bead with a specific DQ2 heterodimer.

definition for complement interference (Figure 3). The results illustrated the complexity and heterogeneity of complement interference and how individual sample and bead were affected. We also contrasted the results from more stringent criteria for complement interference. Although the latter criteria defined the most severe form of complement interference, many samples and beads with moderate but significant interference could have been missed.
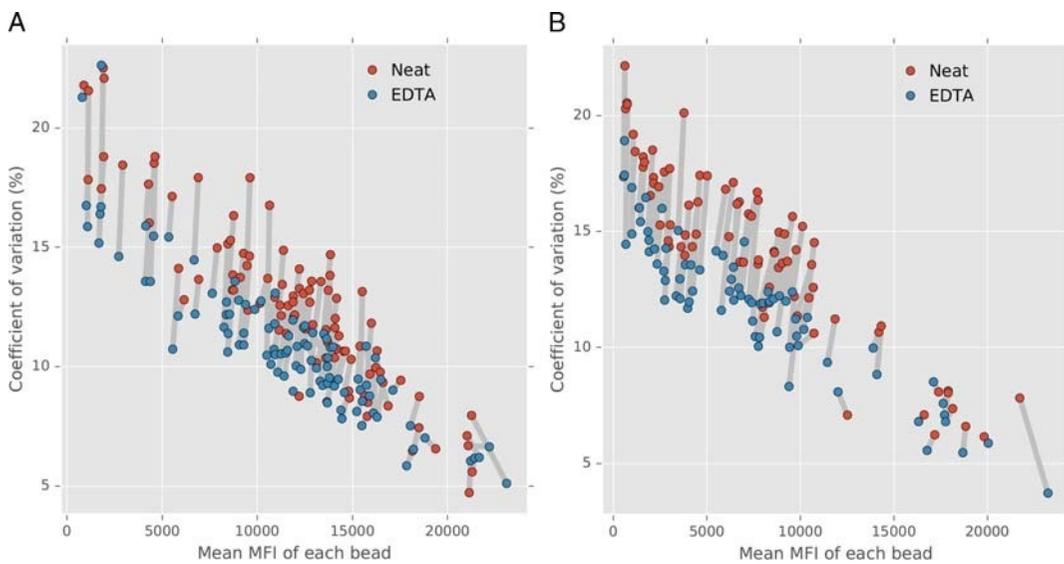
FIGURE 6. The impact of EDTA pretreatment on precision. Figure A and B are for class I and class II beads respectively. A positive control serum pooled from highly sensitized patients were tested with (blue dots) and without (red dots) EDTA pretreatment in parallel over 24 independent runs performed by 5 different technologists. The CV for each bead (y-axis) was plotted against the mean MFI of the corresponding bead (x-axis). The paired data points from the same bead tested with and without EDTA pretreatment were linked by a gray line. Beads with mean MFI lower than 100 were excluded due to inflated CV values.

Using dilution studies at 1:5 and 1:25 we were able to find additional support for most of the complement interference defined by the 4000 MFI increment after EDTA pretreatment. During these studies, the decreasing complement interference was competing with the dilution of the HLA antibody itself, which limited the sensitivity of detecting such interference. EDTA pretreatment may be more sensitive in this regard because it removes the interference without markedly diluting the HLA antibodies. The degree of complement interference varied among samples and individual beads as demonstrated by the patterns seen with dilution (Figure 4). In cases where complement interference could not be supported by dilution, either the interference was below the detection limit of dilution study, or the MFI increment after EDTA pretreatment was due to assay variations.

It is important to note that dilution studies provide information not afforded by EDTA pretreatment.[16] The titer information can be generated with a full titration study, which perhaps provides the most reliable quantitation of antibody strength.[28] Even with abbreviated dilution studies as performed for our study, the dichotomy of MFI trending up or down from 1:5 to 1:25 dilutions may help to separate stronger antibodies from weaker ones when they started with similar MFI values (Figure 4D and H). Therefore, titration or abbreviated dilution studies are indicated if the strength of the antibody may inform clinical decision making or help tracking the response to TPE (Figure 5). The benefit of full titration studies surrounding apheresis was further substantiated by evidence published by Tambur's group recently.[29] One advantage of EDTA pretreatment in conjunction with dilution was that the falsely low MFI values at neat and lower titers were corrected (Figure 5D-F).

Complement interference has been reported to cause sharp fluctuations of serum anti-HLA antibody strength in kidney transplant patients,[30] and questions have been raised about the relevance of complement interference in assay standardization.[8] We showed here an encouraging improvement in precision across a broad spectrum of MFI values with EDTA pretreatment (Figure 6A-B). Although the 2-4% decrease in CV appeared small, most of the beads evaluated had a low to moderate baseline inter-run CVs in the range of 5-20%. Nevertheless, further investigation of this benefit in different settings including inter-laboratory standardization is warranted.

Our study has several limitations. First, EDTA pretreatment may have additional effects on SAB assays other than abolishing complement interference, and unknown substances other than complement factors may affect the assay as well. However, this effect, if any, is likely to be rare given the overall similarity between the 2 methods. Second, not all presumed, weak complement interference can be verified due to the lack of a gold standard. Third, the small number of antibodies that were positive at neat but negative after EDTA pretreatment could be clinically relevant, and the significance of such antibodies remain unclear. Given these concerns, it is prudent to monitor the assay performance in the long-term to document and investigate any discrepancy between a physical (cytotoxicity or flow) crossmatch and a virtual crossmatch. Finally, our samples were primarily from a pretransplant patient population except for the AMR case. Therefore, the effect of transplanation and immunosuppression on the assay and complement interference was not addressed.

In summary, we performed a quantitative evaluation of the impact of EDTA pretreatment on SAB assay in kidney transplant candidates with a broad range of sensitization. We conclude that EDTA pretreatment is suitable for preemptive application on all samples to reduce false negative results and falsely low MFI values. Complement interference is vastly heterogeneous among different patients and single-antigen beads as revealed by EDTA pretreatment. EDTA pretreatment may also be used in combination with dilutions studies if indicated and it has the potential to significantly improve the assay precision.

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