Detection of antibodies to denatured human leucocyte antigen molecules by single antigen Luminex

María Gutiérrez-Larrañaga1,2 | Laura Riesco1,2 | Sandra Guiral1,2 | Juan Irure1,2 | Emilio Rodrigo3 | Javier Ocejo-Vinyals1,2 | Jaume Martorell4 | Jose L. Caro4 | Marcos López-Hoyos1,2 | David San Segundo1,2

1Immunology Department, University Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain
2Tissue Typing Laboratory, University Hospital Marqués de Valdecilla, Santander, Spain
3Nephrology Department, University Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain
4Immunology Department, Hospital Clinic, Barcelona, Spain

Correspondence
David San Segundo, PhD, Immunology Department of University Hospital Marqués de Valdecilla, Valdecilla Avenue s/n, Santander, Spain.
Email: dsanseguido@humv.es

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The anti-HLA antibody detection has been improved in sensitivity and specificity with solid-phase antigen bead (SAB) assays based on Luminex. However, false positive results due to denatured HLA (dHLA) may arise after single antigen test. The aim of this study was to compare the performance of the two Luminex technology-based anti-HLA detection kits available in the market in showing undesired anti-HLA antibody results. A prospective cohort was assessed for anti-HLA antibodies with single antigen A manufacturer (AM) kit and a comparison cohort with single antigen B manufacturer (BM) kit. A total of 11 out of 90 patients in a prospective cohort presented monospecific HLA-I antibodies with AM, and 5 out of 11 confirmed monospecific reaction with BM. Despite the confirmation of monospecific reaction with both manufacturers, 80% were assigned as dHLA reaction by specific crossmatch. Further comparative cohorts detected four out of six monospecific reactions with BM that were confirmed as possible dHLA reactions. A positive SAB test should rule out a reaction against a dHLA molecule, thus avoidance of prolonged waitlist periods or misattribution of anti-HLA reactions after transplantation.

KEYWORDS
antibodies/analysis, cytotoxicity tests, flow cytometry/methods, histocompatibility testing/methods, HLA antigens/immunology, immunologic/methods, microspheres

1 INTRODUCTION

Anti-HLA antibody (Ab) testing experienced a major breakthrough with the development of solid-phase assays based on Luminex technology, increasing the sensitivity and enabling the detection of low levels of HLA-specific Abs.1 Despite this, several potential pitfalls should be taken into account regarding Luminex anti-HLA Ab detection.2 Three different kinds of errors could appear. First, there are false positive results due to Ab binding to misfolded HLA proteins. Thus, denaturing of HLA antigens during the processes of purification and bead

Abbreviations: Ab, antibody; ABMR, antibody-mediated rejection; AM, manufacturer A; B2M, beta 2-microglobulin.; BM, manufacturer B.; CDC, complement-dependent cytotoxicity; dHLA, denatured-HLA; DSA, donor-specific antibodies.; FCXM, flow cytometry crossmatch.; MFI, mean fluorescence intensity.; nHLA, native-HLA; PBMCs, peripheral blood mononuclear cells; SAB, single antigen bead.

Marcos López-Hoyos and David San Segundo contributed equally to this study.
coating could lead to the generation of neo-epitopes that under physiological circumstances would be inaccessible for Abs. Second, lack of cut-off consensus between laboratories and wide intra- and interassay variability could also result in false positive results. Finally, the prozone effect is a widely accepted source of false negative results.3-7

Two types of panels depending on the composition of their target antigen are available. The screening panel has two different bead groups, coated with either HLA class I or class II protein molecules obtained from multiple individual cell lines, and are used as a screening test for the presence or absence of anti-HLA class I/II Abs. However, they do not provide accurate information about Ab specificity. The single-antigen beads (SAB) panel has multiple beads, each one coated with a unique protein molecule representing a single-cloned HLA antigen which enables the identification of any given antibody specificity, and provides the highest degree of anti-HLA Ab resolution. However, denatured beta 2-microglobulin (B2M)-free heavy chains and structurally different HLA class I molecules are present in a single bead which could lead to false positive results,8 whereas denatured-HLA (dHLA) molecule cannot be ruled out in screening panels.9,10

The dHLA reaction has been detected not only in sensitized transplant patients but also in unsensitized males.10 The presence of dHLA reactions was detected in more than 30% of sensitized patients.11 However, there are few reports assessing the clinical impact of the presence of dHLA in the transplant setting.12,13,14

The main objective in anti-HLA Ab testing should be the detection of Abs against a native-HLA molecule. Two manufacturers provide anti-HLA Ab tests based on Luminex technology. Several works have raised differences between them. Some authors have identified a greater proportion of dHLA-I antigens on the surface of beadsets in One lambda SAB kits, whereas Immucor SAB kits present most of its antigens in the native trimeric form composed of HLA-I heavy chain, B2M and peptide, as evidenced by monoclonal Ab binding.15 The main goal of this study was to analyze and compare the performance of the two Luminex technology-based anti-HLA detection kits on showing unexpected anti-HLA antibody results.

2 | MATERIALS AND METHODS

2.1 | Patient selection

The anti-HLA antibody test is performed within routine sampling in patients while listed and after solid organ transplant monitoring. The patients gave their written consent once included on the waiting list. The study was approved by the Local Ethics Committees and conducted in accordance with the Declaration of Helsinki.

2.1.1 | Prospective cohort

A total of 90 serum samples with negative results after anti-HLA Ab screening assay (manufacturer A) were recruited for the study during 1 year in University Hospital Marqués de Valdecilla (Santander, Spain). Forty out of 90 (44.4%) sera came from solid organ transplant candidates newly included in the institution’s waiting list whereas 50 (55.5%) sera came from solid organ transplant patients followed posttransplant within 1 year. A subsequent assessment of samples rendered negative after screening but positive on SAB-assay results were selected for further analysis (ie, acid treatment of the beads, CDC and flow cytometry crossmatch [FCXM] as described below). Finally, 11 sera with confirmed negative screening results but positive monospecific anti-HLA class I results were selected. Their main demographic and immunological parameters are summarized in Supporting Information, Table S S1. The 11 samples were chosen in order to facilitate the selection of donor HLA-typed cells for testing each monospecific HLA reactive sera.

In order to elucidate if such findings may be differentially affecting the performance of the kits on detecting unexplained anti-HLA results on SAB assays, we reanalyzed them with B manufacturer’s anti-HLA Ab kit.

2.1.2 | Comparison cohort

Serum samples from six patients with confirmed negative anti-HLA screening and positive anti-HLA class I monospecific reaction in SAB assay (manufacturer B) not explained by previous sensitization events were selected in the Clinic Hospital (Barcelona, Spain) and reanalyzed using AM kit. The main demographic and immunological parameters of the patients for potential allosensitization are summarized in Table SS2.

An overview of the study is detailed in Figure SS1.

2.2 | Anti-HLA antibody test

The presence of anti-HLA Abs was tested by using screening and SAB assays from the only two manufacturers available as described in the text (LABScreen Mixed, Cat LSM12, Lot: 020; LABScreen Single Antigen
Class I, Cat LS1A04, Lot: 010; and LABScreen Single Antigen Class II, Cat LS2A01, Lot 012, One Lambda, Canoga Park, California) referred as A manufacturer; and (Lifescreen Deluxe, Cat LMX, Lot 3 006 878; LSA class I, Lot 3 006 346 and LSA class II, Lot 3 006 494, Immucor, Gateway Drive, Georgia) referred as B manufacturer and following manufacturers' instructions; and analyzed on a Luminex platform (LabScan100). The cut-off value was set at 500 baseline mean fluorescence intensity (MFI) for screening and 1500 baseline MFI for SAB test. Such a difference in the cut-off is intended to minimize false negative results in anti-HLA Ab screening and false positive results in SAB assays. We considered sera with values between 1500 and 3000 baseline MFI as weakly positive.

2.3 | In vitro anti-HLA reactivity against denatured vs native HLA-molecule

In order to decipher the reaction against dHLA or native-HLA (nHLA) molecule, in samples analyzed with AM kit rendering negative screening but positive SAB results, acid treatment of the SAB beadsets was performed as previously described. Briefly, the beads were treated with 1 mL of 1% bovine serum albumin and 0.1 M glycine buffer (pH = 2.8) during 30 minutes, then washed twice with the wash buffer provided by manufacturer prior to sera incubation and subsequently followed the manufacturer's instructions. The denatured beads were confirmed with anti-w6/32-phycoerythrin monoclonal antibody (Biolegend, San Diego, California), a representative example of acid treatment of the beads is shown in Figure SS2). Because there is no consensus for a cut-off value of MFI after acid treatment, we decided to set at 1500 MFI. Values between 1500 and 3000 MFI were considered as weakly positive. The MFI values pre- and postacid treatment are described in Table SS3.

2.4 | Ex vivo anti-HLA reactivity

Cytotoxic (CDC) and FCXM were performed when possible in samples with negative results for screening while positive SAB results using A manufacturer kit against reactive HLA-typed cells. CDC was conducted as previously described. For FCXM, peripheral blood mononuclear cells (PBMCs) isolated by Ficoll gradient were incubated with rat serum (Sigma Aldrich, St. Louis, Missouri) during 20 minutes to avoid unspecific binding (Cytometry A. 2016;89 (1):1001-1009). Undiluted and 1/10 diluted serum was added to PBMCs and incubated during 30 minutes at room temperature. Anti-CD3 ECD (clone UCHT-1, Beckman Coulter, Brea, California), CD19 PC5 (clone J3-119 Beckman Coulter) and subsequently Fab'-IgG FITC (Immunostep, Salamanca, Spain) were added and samples were acquired using a Navios EX Flow Cytometer (Beckman Coulter). Flow cytometry crossmatch for class I antigens was considered positive in the CD3+ gate when the ratio of median fluorescence value serum/median fluorescence value negative control was >1.5; for class II antigens, the FCXM was considered positive in CD19+ gate when the ratio was >2.0.

The HLA typing of the donors was performed for HLA loci A, B, DRB1, DQA1 and DQB1 by high-resolution sequence-specific primer (Life Technologies, Brown Deer, Wisconsin) to confirm reaction against nHLA.

2.5 | Statistical analysis

Kolmogorov-Smirnoff test was used to show if the data of baseline MFI followed a normal distribution. The Mann-Whitney test was used to compare median baseline MFI. All tests were performed using GraphPad software version 7.0 (San Diego, California). The R software and “ggplot2” package have been used for graphics.

3 | RESULTS

3.1 | Single antigen positive reactions against denatured or native HLA molecules

A total of 21 patients from a prospective cohort (23.3%) showed positive SAB results (both in HLA class I and class II specificities) with negative screening test result. For further analysis, we focused only on the 11 patients (9.9%) with positive reactions only against HLA class I for simplicity. Five out of 11 (45.4%) were transplanted patients, although none of the monospecific reactions were donor-specific. Of note, only two samples were from unsensitized males. A summary of potential sensitization of the patients is summarized in Table S S1. Upon acid treatment, the HLA antigens attached to the beads were denatured; a negative result after SAB of acid-treated beads would suggest a nHLA reaction. However, a total of 11/11 (100%) patients had positive reactions after bead-treatment (Figure 1), so dHLA reaction could not be ruled out. Moreover, to confirm the reactivity of the anti-HLA Abs, both CDC and FCXM against a limited number of specific HLA-typed cells were performed. As expected, all the samples with potential anti-dHLA
3.2 Comparison of baseline MFI from screening vs single antigen test

The SAB test was performed with sera from a prospective cohort with a previous negative result on screening test. Once a positive result in HLA class I was found, we calculated the mean baseline MFI of the bead groups where the positive HLA antigen was included (i.e., A*11:01 is present in beads #008, #014, #015 and #016 in the screening full panel of Lot 020 from One Lambda and in bead#CLI02, #CLI04, #CLI05, #CLI06 panel of Lot

**FIGURE 1** Mean fluorescence intensity (MFI) values in single antigen bead and acid-treated bead assays. The MFI values after single antigen bead (SAB) assay detecting monospecific reactions in each patient are shown (A). After acid treatment of the beads as described in Material and Methods section, an increase in MFI values is observed (B). The cut-off value in SAB assay was set at 3000 MFI (black line) and in acid-treated bead assay was set at 1500 MFI (dotted line). The arrows in each patient indicate the MFI value of each monospecific reaction after acid treatment of the beads.

**FIGURE 2** Comparison of median MFI for SAB Class I (white circles) and screening (black squares) test for anti-HLA antibodies in serum with monospecific reactions.

reactions that could be tested in crossmatch rendered both CDC and FCXM negative results, summarized in Tables 3 and 4.
3 006 878 from Immucor). Figure 2 describes the MFI values obtained after screening and single antigen test of the sera.

### 3.3 Confirmation of nonconcordant results with B manufacturer’s kit

In order to clarify the monospecific reactions observed with A manufacturer (AM), 11 serum samples of patients with confirmed negative screening results and positive anti-HLA class I monospecific reactions with AM kit were selected for further analysis using B manufacturer’s (BM) kit. Seven out of the 11 serum samples (58.3%) continued displaying negative screening but positive anti-HLA class I SAB-assay results, although a significant reduction of median (interquartile range) baseline MFI levels (AM vs BM) 3877 (3633-6101) vs 1405 (100-2555), was observed ($P < .0001$). In this comparative study, the anti-HLA reactions of samples rendering negative crossmatch results were considered to be directed against dHLA antigens on the beads. The comparative results between the two manufacturers are summarized in Table 1.

### 3.4 Monospecific reactions observed with B manufacturer—comparison cohort

Six serum samples from the comparison cohort were found to repeatedly show negative screening but positive monospecific anti-HLA class I SAB-assay results when analyzed employing BM kit. One out of six (16.7%) confirmed such results by analysis using AM kit, while the other five rendered negative results in both screening and SAB assays with AM kit. The comparative results between the two manufacturers are summarized in Table 2.

#### TABLE 1
Comparative results of samples from the prospective cohort

| Serum # | Class I bead | AM     | BM     | Reaction | Crossmatch CDC/FCXM | Assignment |
|---------|--------------|--------|--------|----------|----------------------|------------|
| 1       | B7           | 3633   | 3326   | $T_R$    | NEGATIVE             | Denatured  |
| 2       | A23          | 3877   | 2555   | $T_R$    | NEGATIVE             |            |
| 3       | B17          | 6100   | 1321   | $F_{RA}$ | NEGATIVE             | Denatured  |
| 4       | A34          | 3861   | <100   | $F_{RA}$ | NA                   |            |
| 5       | B73          | 13 574 | 3199   | $T_R$    | NA                   |            |
| 6       | A11          | 5645   | <100   | $F_{RA}$ | NEGATIVE             | Denatured  |
| 7       | Cw7          | 3572   | 1583   | $T_R$    | NEGATIVE             | Denatured  |
| 8       | A80          | 10 139 | 1405   | $F_{RA}$ | NA                   |            |
| 9       | Cw9          | 5190   | <100   | $F_{RA}$ | NA                   |            |
| 10      | B37          | 2050   | 1617   | $T_R$    | NA                   |            |
| 11      | A34          | 3821   | <100   | $F_{RA}$ | NA                   |            |

**Note:** Bold text mean fluorescence intensity (MFI) of monospecific reactions. Abbreviations: AM, manufacturer A; BM, manufacturer B; FRA: false reaction A manufacturer; NA, not available; $T_R$, true reaction (confirmed in both manufacturers).

#### TABLE 2
Comparative results of samples from comparison cohort

| Serum # | Class I bead | AM     | BM     | Reaction | Crossmatch CDC/FCXM | Assignment |
|---------|--------------|--------|--------|----------|----------------------|------------|
| 12      | A23/A24      | 206    | 3690   | $F_{RB}$ | NEGATIVE             | Denatured  |
| 13      | A23/A24      | 66     | 1787   | $F_{RB}$ | NEGATIVE             | Denatured  |
| 14      | A23/A24      | 396    | 7478   | $F_{RB}$ | NEGATIVE             | Denatured  |
| 15      | A23/A24      | 397    | 3091   | $F_{RB}$ | NEGATIVE             | Denatured  |
| 16      | B57/B58      | 92     | 2005   | $F_{RB}$ | NA                   |            |
| 17      | B57/B58      | 5961   | 3055   | $T_R$    | NA                   |            |

**Note:** The monospecific reaction against A23/A24 and B57/B58 refers to A9 and B17 reactions. The mean fluorescence intensity (MFI) values are the mean of MFI from the different alleles in each single antigen panel. Bold text mean fluorescence intensity (MFI) of monospecific reactions. Abbreviations: AM, manufacturer A; BM, manufacturer B; $F_{RB}$: false reaction B manufacturer; NA, not available; $T_R$, true reaction (confirmed in both manufacturers).
4 | DISCUSSION

The anti-HLA Ab test is mandatory prior to any solid organ transplantation in order to quantify the level of allosensitization. The SAB-assay itself has several pitfalls and a proper monitoring of anti-HLA Abs should be performed while the patient is included on the waiting list. The guidelines indicate monitoring every 3 months in all solid organ waiting list patients. Patients that have lost a previous transplant, women potentially sensitized due to pregnancy or abortions and transfused patients are potential candidates to identify reactions against HLA antigens. Our idea was to better identify this kind of patients by performing directly SAB assay as first option instead of anti-HLA Abs screening. However, an increased number of missassigned-HLA specificities could lead to unacceptable mismatches with potential donors and prolonged periods on waiting list. Moreover, testing anti-HLA Abs by SAB assays in transplant monitoring could assign a patient with donor-specific anti-HLA antibodies (DSA), when the identified antibodies could actually be directed against dHLA, with uncertain impact on the allograft. The interference with complement and prozone effect could be avoided with serum ethylenediaminetetraacetic acid-treatment.

Deciphering the reactivity of the anti-HLA Abs between denatured or native HLA would help in the identification of deleterious anti-HLA Abs, with different implications if on waiting list or posttransplant monitoring. In the first case, it would potentially increase the time on waiting list, whereas in transplanted patients, humoral rejection diagnosis requires a confirmatory biopsy with circulating DSA. Thus, the identification of the found antibodies as directed against dHLA would avoid treatment of nonrelevant DSA. However, with clinical and/or histological findings, other means of humoral rejection mediated by non-HLA Abs (MHC class I chain-related sequence A, angiotensin II type 1 receptor, endothelial cells and others) should be ruled out.

The presence of anti-HLA Abs in patients not exposed to any allosensitizing event due to cross-reaction with viral epitopes has been documented. However, the literature is mixed with recent reports identifying no cross reactivity with viral and allo antibodies.

For this reason, a proper assessment of the nature of these antibodies is of great importance for their clinical implication. Pretransplant anti-dHLA DSA reactions have been associated with negative FCXM, low risk of acute antibody-mediated rejection (ABMR) and better long-term graft survival. It should be taken into account the different anti-HLA detection level of FCXM and SAB assay. In our study, no clinical assessment was performed but recently kidney transplant recipients with anti-dHLA-Cw DSA showed negative FCXM reactions, lower ABMR rate and better graft survival. The HLA-Cw is expressed at a lower frequency on cells used within FCXM, but is at a high density on the SAB assay. This could lead discrepant results. In our study, most of the monospecific reactions selected had more than 3000 MFI with a negative FCXM which supports the reaction against dHLA. However, a limitation of the work is that, in order to show reaction against dHLA, only acid treatment in prospective cohort was performed. Previously, several groups also confirmed the assessment with iBeads with good correlation with acid treatment.

In patients waiting for heart or lung transplantation, the definition of forbidden HLA antigens should be accurate due to virtual crossmatch and lack of prospective crossmatch performance. Cao et al have performed direct SAB in lung-waiting list patients and identified more anti-HLA antibodies compared with screening assays in samples rendering negative crossmatches. These observations are consistent with our findings; however, the screening and SAB assays have different source of HLA molecules coating the beads, the screening panel is manufactured from nonrecombinant cell line whereas SAB panel with recombinant cell lines denaturing differently and present on the surface of the beads at different densities providing potential discrepant results.

Since there are two different manufacturers of SAB assay for anti-HLA antibody testing, several studies have compared the results provided by both of them, with no substantial differences. Some discordant results could be explained by different HLA-load constructs on the beads. A recent study compared the performance and accuracy of the only two commercially available Luminex-based SAB assays in the ABMR setting. Authors concluded that both kits, despite significant differences in their sensitivity and specificity, had a good correlation.

Our results show that most of confirmed monospecific HLA class I reactions from unsensitized patients by AM and BM render both CDC and FCXM negative results that point to dHLA reactions and could be due to similar way of HLA antigen-load on beads in their manufacturer.

In the present study, all monospecific reactions detected by SAB, irrespectively of baseline MFI levels, render negative CDC and FCXM suggesting dHLA reactions. Although it should be taken into account that the different sensitivity of the techniques could justify some discordant results between SAB test and crossmatch.

Upon these results, we propose that a positive monospecific reaction detected by SAB in a patient without history of sensitizing events should be defined as nHLA, after a negative reaction with acid treatment of the beads,
CDC and/or FCXM against donor cells with the type of interest, and, besides the sample should be studied with the kit of the other manufacturer, which could have logistic problems in laboratory organization.

The main limitation of this study is the scarce number of clinical cases to discuss after transplantation; although the study was driven, in part, from a technical laboratory point of view. The results were performed in a single-mixed beads lot; however, the confirmation in different bead lots would improve the robustness of our results. Another limitation was the lack of CDC and FCXM for all anti-HLA reactivities detected, most of them due to their low frequency in our population (A*34:0-1.5%, B*73: 0-2% and A*80: 0-2% of European Caucasians).29 Moreover, the identification of Abs against low frequency HLA could suggest false positive reactions.

To better rely on anti-HLA Abs tests the efforts tests, efforts should identify potential biases in anti-HLA Ab results, leading to the fine-tuning of anti-HLA Ab profiles of the patients which could be of special importance in those who are highly sensitized.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request

ORCID
David San Segundo 𝓞 https://orcid.org/0000-0001-5745-9072

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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