

## Luminex-based virtual crossmatching for renal transplantation in South Africa

Catherine M Worsley, Elizabeth S Mayne, Melinda S Suchard

**Background.** Current practice in the Johannesburg renal transplantation programme is to perform a transplant when the patient's complement-dependent cytotoxicity and flow cytometric crossmatches are negative. However, even in patients with negative crossmatches early graft rejections have occurred. We retrospectively evaluated the use of Luminex anti-human leukocyte antigen (HLA) antibody detection technology, often termed 'virtual crossmatching', compared with the flow cytometric crossmatch, for predicting graft outcome in renal transplant patients.

**Methods.** Sixty-four recipients were crossmatched against multiple donors during their routine work-up for transplant (111 crossmatches); 17 of these patients received transplants during the study period. Anti-HLA antibody detection was performed using Luminex technology and the results were compared with the flow cytometric crossmatch results and with short-term graft success.

**Results.** Compared with flow cytometric crossmatch results, the sensitivity and specificity of Luminex virtual crossmatching was 85.7% and 90.7% for the T-cell crossmatch and 100% and 87.2% for the B-cell crossmatch. Both the sensitivity and specificity of Luminex for predicting short-term graft success were 100%.

**Conclusions.** Strong evidence is provided that single-antigen assays provide improved sensitivity to detect clinically relevant anti-HLA antibodies and can reliably be used to predict short-term graft success. We recommend incorporation of single-antigen Luminex methodology into the routine work-up algorithm of renal transplant recipients in South Africa.

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### The role of anti-HLA antibodies in renal transplantation

Human leukocyte antigens (HLA) are a highly polymorphic system of glycoproteins that have a functional role in the presentation of peptides to the immune system.<sup>1,2</sup> HLA class I molecules are expressed on most nucleated cells, while HLA class II antigens are generally found on professional antigen-presenting cells.<sup>3</sup> HLA class II antigens can also be upregulated on kidney cells.<sup>4</sup> As a highly polymorphic system, however, specific HLA alleles can become the targets of antibody responses in people sensitised to foreign HLA molecules during pregnancy, transfusion of blood products, or organ transplantation.<sup>1,5,6</sup> Humoral responses or sensitisation to HLA are major barriers to solid organ transplantation.<sup>1,7</sup> Sensitised patients are more likely to crossmatch positive with potential donors and have a reduced chance of receiving a renal transplant.<sup>8</sup> The detection of anti-HLA antibodies helps to predict both the function and survival of transplant allografts.<sup>9</sup> Most allograft damage is mediated through the activation of complement; C4d deposition is widely accepted as a marker for antibody-mediated rejection in renal allografts.<sup>10</sup>

### Detection of anti-HLA antibodies

In the Johannesburg renal transplantation programme, recipient placement on the waiting list for cadaveric kidney allocation is determined by a composite score comprising length of time on the waiting list, recipient age, and the percentage of panel-reactive

antibodies (PRAs, an estimate of the percentage of HLA types in the general population against which the recipient has antibodies). Laboratory testing before transplantation includes crossmatching donor cells (lymphocytes as surrogates of renal cells) with recipient serum. If a recipient has antibodies against a donor's HLA type, the antibodies will bind to the donor's cells and the crossmatch should be positive, whereas if the recipient does not have anti-HLA donor-specific antibodies, the crossmatch should be negative. The Johannesburg renal transplant programme currently performs a transplant based on both a negative complement-dependent cytotoxicity (CDC) and the more sensitive flow cytometric crossmatch. However, both can be subject to interference,<sup>8,11</sup> and even if both crossmatches are negative, early graft rejections still occur. Higher organ rejection rates occur among patients with donor-specific anti-HLA antibodies prior to transplantation.<sup>9</sup> Re-evaluation of the algorithm of laboratory-based testing is therefore critical to provide a comprehensive work-up before transplantation.

Acute allograft rejection is currently diagnosed by percutaneous needle biopsy of the renal allograft.<sup>12</sup> Acute antibody-mediated rejection is a sequential process; initial endothelial damage by donor-specific anti-HLA antibodies is followed much later by histological alterations (such as C4d deposition and significant interstitial infiltration) and clinical manifestations (graft dysfunction and rejection).<sup>12-14</sup> Detecting donor-specific anti-HLA antibodies has several clinical applications, e.g. predicting transplant success<sup>1,11,15,16</sup> and helping to streamline organ allocation particularly for highly sensitised patients (who often crossmatch positive against multiple donors and spend long periods on waiting lists).<sup>1,14</sup>

### Single-antigen technology is used to detect HLA antibodies

The Luminex anti-HLA antibody detection assay is reportedly more sensitive and specific than either the CDC or flow cytometric crossmatches.<sup>5,9,15</sup> In fact, some consider the Luminex antibody detection technique to be the new gold standard for identifying anti-HLA antibodies.<sup>6</sup> This technique is a solid-phase assay in which purified HLA molecules (either of a single HLA type or a

Department of Haematology and Molecular Medicine, Faculty of Health Sciences, University of the Witwatersrand and National Health Laboratory Services, Johannesburg

Catherine M Worsley, BSc, BSc Hons, MSc

Elizabeth S Mayne, MB BCH, FCPATH (Haem), MMed Path (Haem)

Melinda S Suchard, BSc, FCPATH (SA) (Clin), MMed (Clin), DTM&H

Corresponding author: C M Worsley (catherine.worsley@nhls.ac.za)

combination of types) are attached to beads. These molecules will bind to anti-HLA antibodies in the patient's serum.<sup>1,15</sup> Using single-antigen technology, the Luminex technology can predict a patient's sensitisation to particular HLA types prior to transplantation without performing a physical CDC or flow cytometric crossmatch (termed a 'virtual crossmatch').<sup>5,6,15</sup>

In this retrospective study, we compared Luminex virtual crossmatch predictions with the flow cytometric crossmatch results. In the patients who received transplants, Luminex virtual crossmatch predictions were also compared with short-term (6-month) renal graft success. The importance of technological advances in laboratory-based testing in assessing the risk of antibody-mediated rejection in renal transplantation is highlighted.

## Objectives

We aimed to determine whether Luminex anti-HLA antibody analysis could accurately predict flow cytometric crossmatch outcomes and whether Luminex antibody detection could predict clinical short-term graft success. We hypothesised that in identifying donor-specific anti-HLA antibodies before transplantation, this solid-phase approach could ensure more efficient organ allocation and accurately determine acceptable donor-recipient combinations.

## Materials and methods

Sera were obtained from 64 recipients who were routinely crossmatched against either cadaveric or related living donors (September 2009 - August 2010). These were stored at -70°C prior to antibody testing. Ethics approval for this study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (HREC M090331). Donor HLA typing was performed by the South African National Blood Service as routine patient work-up.

Luminex single-antigen beads coated with HLA antigens (Gen-Probe, USA) were incubated with recipient sera in a 96-well plate in the dark on a rotating platform. After washing, phycoerythrin-conjugated goat anti-human IgG (Gen-Probe, USA) was added which would bind to recipient HLA antibodies captured on the beads. The test and control samples were then acquired using the Luminex Flow Analyzer and Luminex<sup>®</sup> 100 IS™ version 2.3 software (Luminex, USA). The Quicktype for Lifematch 2.5.2 software (Gen-Probe, USA) was used to interpret results. All assays included manufacturer's positive and negative control sera and were prepared according to the Gen-Probe, USA, instructions.<sup>17</sup>

To determine whether an individual single-antigen bead was positive (i.e. if recipient anti-HLA antibody was bound to the bead), the mean fluorescence intensity (MFI) of the bead was divided by the MFI of the three negative control beads. The background adjustment factor was subtracted from this value to provide three different adjusted values. A bead was considered to be positive if two or more of the adjusted values were positive.<sup>17</sup> MFIs of ~1 000 or more were generally considered as positive, although each case was analysed individually and pattern reactivity was also taken into consideration; three recipients had antibody testing performed on their post-transplant sera following documented organ rejection.

Recipient sera were routinely crossmatched with ABO blood-group-compatible donor T and B lymphocytes, with 111 T- and B-cell flow cytometric crossmatches being performed between 64 recipients and 27 donors (related living and cadaveric). Some recipients were crossmatched against multiple donors. Donor lymphocytes were incubated with recipient sera for 1 hour at 37°C. Donor cells were also incubated with appropriate positive (multispecific and anti-lymphocyte globulin) and negative (AB

serum and autologous donor serum) control sera. Cells were then washed, and were incubated with rabbit anti-human IgG (Fab')<sub>2</sub> (FITC) (Dako, Denmark) for 30 minutes at 4°C to detect any bound anti-HLA antibodies. Donor lymphocytes were then washed and stained with CD19 PE (Beckman Coulter, France) and CD3 APC (BD Biosciences, USA) to stain the B and T cells, respectively. After a 15-minute incubation, 300 000 events were acquired on the LSRII flow cytometer (BD Biosciences, USA). Results were analysed using FlowJo 8.5.2 software (Treestar, USA). B-cell crossmatches were considered positive if the MFI was at least 5 times greater than the MFI of the lowest negative control. T-cell crossmatches were considered positive if the MFI was at least 1.5 times greater than the MFI of the lowest negative control.

Sensitivity, specificity, and positive and negative predictive values of the Luminex virtual crossmatch were retrospectively calculated in comparison with the flow cytometric crossmatch and with short-term graft outcomes. A Mann-Whitney test was performed to compare the MFIs of single-antigen beads between positive and negative flow cytometric crossmatches.

## Results

### Luminex anti-HLA antibody testing compared with flow cytometric crossmatch results

For 111 crossmatches performed on 64 recipients, the sensitivity, specificity, positive and negative predictive values for predicting the T-cell crossmatch were 85.7%, 90.7%, 57.1% and 97.8%, respectively, and for the B-cell crossmatch, 100%, 87.2%, 12.5% and 100%, respectively (Table I).

We compared the MFIs of the single-antigen beads (which predicted the flow crossmatch as positive) with actual positive or negative crossmatch results for both T (class 1) and B (class 2) cell flow cytometric crossmatches (Fig. 1). This was done to try to predict whether an MFI cut-off could be used to predict flow cytometric crossmatch outcomes. For the class 1 crossmatch, the MFIs of the single-antigen beads were significantly greater for the positive compared with the negative flow cytometric crossmatches ( $p < 0.0001$ ). Similarly, the MFIs of the single-antigen beads were significantly increased in the positive compared with the negative class 2 flow cytometric crossmatches ( $p = 0.0007$ ) (Fig. 1). However, although the MFIs are significantly different between positive and negative crossmatches for both class 1 and 2, there is some overlap in antibody reactivity between positive and negative flow cytometric crossmatches, which makes it difficult to determine an exact MFI cut-off for predicting flow cytometric crossmatch positivity.

### Relevance of HLA antibody testing to clinical outcome

Seventeen of the 64 recipients received transplants (all B- and T-cell flow cytometric crossmatch-negative), and graft success was compared with the predictive antibody screening results. Fourteen of the 17 (82.4%) patients had successful transplants, which correlated with the Luminex prediction as HLA antibodies were not detected in these patients (Table II). However, 3 of the 17 recipients (17.6%) experienced acute rejection and graft failure/loss (Tables II and III). For each of these 3 patients, although the flow cytometric crossmatch was negative, Luminex analysis revealed the presence of donor-specific anti-HLA antibodies in both pre- and post-transplantation sera (Table III). Luminex antibody testing was accurate in predicting short-term graft success, with both a 100% sensitivity and specificity, as well as 100% positive predictive value (PPV) and negative predictive value (NPV) values (Tables II and III).

**Table I. Evaluation of the Luminex virtual crossmatch**

	N	TP	TN	FN	FP	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
T-cell crossmatch (HLA class 1)	111	12	88	2	9	85.7	90.7	57.1	97.8
B-cell crossmatch (HLA class 2)	111	2	95	0	14	100	87.2	12.5	100

TP = true positive; TN = true negative; FN = false negative; FP = false positive; PPV = positive predictive value; NPV = negative predictive value.

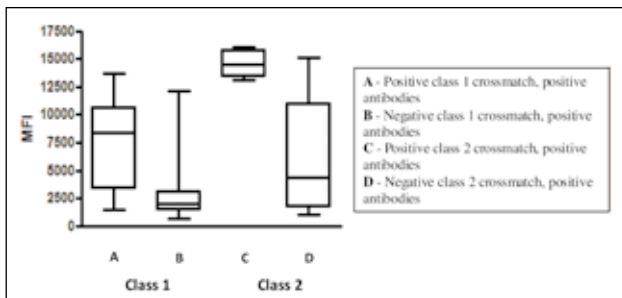


Fig. 1. Single-antigen MFIs are significantly different between positive and negative flow cytometric crossmatch for class 1 and 2.

**Discussion**

Patients who have donor-specific anti-HLA antibodies before transplantation have poor graft outcomes, with the vast majority of cases of antibody-mediated renal graft rejection attributed to anti-HLA antibodies.<sup>1,13,18</sup> Solid-phase assays such as Luminex antibody testing can sensitively and specifically detect these

antibodies and predict graft success,<sup>1,11,15,16</sup> and may be adequate to replace older crossmatch methodologies.<sup>2,19</sup> Our primary objective was to determine whether Luminex antibody analysis could accurately predict T- and B-cell flow crossmatches for patients awaiting renal transplantation in our setting. We used single-antigen technology which detects antibodies directed at a single HLA allele in highly sensitised individuals.

Compared with the flow cytometric crossmatch, the Luminex virtual crossmatch had a sensitivity of 85.6% for prediction of the T-cell crossmatch and a sensitivity of 100% for prediction of the B-cell crossmatch. The sensitivity for prediction of the crossmatch was lower than reported,<sup>5,8,11,20</sup> and we subsequently compared Luminex results with graft outcomes. Although the PPV for the Luminex virtual crossmatch was only 57.1% compared with the T-cell flow cytometric crossmatch, the PPV in comparison with transplant outcome was 100%. The Luminex antibody detection assay more reliably predicted graft failure over this period than the conventional flow cytometric assay.

**Table II. Comparison of Luminex antibody predictions with renal graft success**

N	TP	TN	FN	FP	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
17	3	14	0	0	100	100	100	100

TP = true positive; TN = true negative; FN = false negative; FP = false positive; PPV = positive predictive value; NPV = negative predictive value.

**Table III. Renal allograft rejection cases**

	Case 1	Case 2	Case 3
Patient history	31-year-old male	31-year-old male Previous transplant rejection	50-year-old female
Living related or cadaver donor	Living related	Cadaver	Cadaver
Post-transplant time to rejection	<2 weeks	<5 days	<2 days
Nephrectomy	Yes	Yes	Yes
Histological diagnosis	Acute rejection (C4d staining negative)	Acute humoral rejection (C4d staining positive)	Delayed hyperacute vascular rejection (C4d staining not performed)
Donor HLA type	A*03,32 B*15,44 DR*04(53),16(51)	A*02,24 B*07,35 DR*04(53),15(51)	A*02,11 B*07,15 DR*04(53),13(52)
Luminex antibody results and strengths (MFI)	DR*51 (pre 6 342, post 3 169)	A*02 (pre 1 279, post 848) B*07 (pre 1 443, post 2 124) DR*04 (pre 1 439, post 1 778)	B*15 (pre 1 936, post 1 745) DR*04 (pre not detected, post 3 344)

pre = pre-transplantation; post = post-transplantation.

The MFIs of the single-antigen beads (which predicted positive-flow cytometric crossmatches) were compared between the actual positive- and negative-flow crossmatch results. Although the MFIs were significantly different ( $p < 0.001$  for class 1 and  $p = 0.0007$  for class 2), there was some overlap in antibody reactivity that cannot be ignored. This makes it difficult to define an absolute Luminex MFI cut-off value for predicting flow cytometric crossmatch positivity.

Although anti-HLA antibody detection assays have been criticised as being over-sensitive,<sup>15,20</sup> our study did not demonstrate this with the Luminex assay showing an NPV of 100% (14 patients who were successfully transplanted showed no donor-specific anti-HLA antibodies). All antibodies, including 'weak' antibodies, were clinically relevant. These findings support addition of Luminex technology to routine pre-transplantation work-up and possibly supplanting the flow cytometric crossmatch assay in our laboratory. It is not clear why the flow cytometric crossmatch was negative for the 3 patients whose grafts were rejected, but the assay is subject to methodological and biological interferences such as poor donor lymphocyte viability, interfering therapeutic antibodies, auto-antibodies, and non-HLA-specific antibodies confounding results.<sup>21,22</sup> The flow cytometric crossmatch also depends on the varying levels of skill of staff members performing the assay.<sup>22</sup> Since only patients who have negative crossmatches are given transplants, the PPV or NPV of the flow cytometric assay compared with graft success cannot be reported.

Other advantages of implementation of Luminex single-antigen technology include post-transplantation monitoring to predict and evaluate graft dysfunction and the ability to target specific donor-directed anti-HLA antibodies in desensitisation regimens.<sup>1,23</sup> The Luminex anti-HLA antibody assay also has the advantage of not requiring viable donor cells, can be performed at convenience (before the time of transplant in the case of cadaveric donation), and specifically detects HLA-directed antibodies.<sup>15</sup>

## Conclusions

In this South African cohort, virtual crossmatching using Luminex single-antigen beads for detection of anti-HLA antibodies predicted clinical outcome better than flow cytometric crossmatching. Luminex technology should be routinely implemented into pre-transplantation work-up of renal transplant recipients.

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**Authors' contributions.** CMW and MSS designed the study. CMW performed the laboratory work, data analysis, and prepared the manuscript. ESM and MSS aided in preparing and reviewing the manuscript.

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