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Original article

Assessment of Luminex Mean Fluorescence Intensity Values with Complement-Dependent Cytotoxicity Results in Detection of Antibodies Against Human Leucocyte Antigen

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Abstract

Introduction. Antibodies to human leukocyte antigens (HLA) are one of the major immune barriers to successful organ transplantation. In addition to complement-dependent cytotoxicity (CDC) assay as a standard method for the HLA antibodies detection, more sensitive solid-phase assays like Luminex were introduced. The aim of this study was to define mean fluorescence intensity (MFI) cutoff values of HLA Class I antibodies detected by Luminex from those detected by CDC given the possibility to use only Luminex assay in HLA antibody screening.

Methods. This is a retrospective analysis of the HLA antibody screening results of patients on a kidney transplant waiting list, performed at the Tissue Typing Laboratory, Clinical Hospital Center Rijeka, Croatia, from January 2012 to July 2019. The study included 1,665 sera tested in parallel by CDC and Luminex techniques.

Results. Almost half sera contained HLA antibodies (47.9%), significantly more detected by the Luminex than a CDC method. Antibodies against HLA-A and HLA-B molecules had higher MFI values, relative to the HLA-C antibodies, as well as antibodies detected by Luminex and CDC than those detected by Luminex alone. A cutoff MFI $\geq 9,204$ for Luminex detected HLA Class I antibodies correlated with positivity in the CDC assay. Besides MFI, several factors need to be taken into consideration in interpreting test results to identify unacceptable antigen mismatch.

Conclusion. The results of this analysis suggest that the current features of the Luminex technique provide the most benefit in the HLA antibody screening when combined with other methods, like CDC.

Keywords: Antibodies, CDC Test, HLA antigens, Kidney Transplantations, Luminex

Introduction

Kidney transplantation is the best therapy for patients suffering from end-stage renal disease [1]. The most important immune factors that affect graft survival are the ABO blood group system and the human leukocyte antigen (HLA) system [2,3].

The polymorphism of HLA system always causes some degree of mismatch (MM) between the donor and the transplant recipient, except in identical twins [4]. Donor HLA antigens that the recipient does not have induce cellular or humoral immune response. Today in solid organ transplantations, the cellular response can be controlled by immuno-suppressive therapy, while the humoral response still constitutes a major cause of immune-mediated graft rejection [5]. Therefore, HLA antibody monitoring in the recipient and determining unacceptable antigens is an important prerequisite for a successful transplantation [6].

HLA alloimmunization can result from prior contact with foreign HLA antigens via blood, after tissue or organ transplantation, transfusion of blood products and/or pregnancy [7].

Basic methods for detecting the presence of HLA antibodies are screening of patients' serum and crossmatch between recipients' serum and donor lymphocytes. The "gold standard" in an HLA antibody detection for many decades was a complement dependent cytotoxicity test (CDC) assay [8]. The knowledge of clinical relevance of HLA antibodies in organ transplantation has prompted the development of new techniques, since one of the major drawbacks of CDC method was its low sensitivity. By modifying cell-based assays, the highest sensitivity was achieved by flow cytometry, while in the 90s, solid phase assays (SPA) like Enzyme-Linked Immunosorbent Assay and, currently the most sensitive, Luminex technique have been introduced [9].

Luminex technology became an important technique in HLA antibody screening, significantly reducing the use of CDC method. Some laboratories decided to exclude CDC method out of the HLA antibody screening and to rely solely on single antigen bead (SAB) assays [10].

The aim of this study was to assess median fluorescence intensity (MFI) cutoff values of HLA antibodies detected by Luminex only from those detected by both, Luminex and CDC assays in the light of possible replacement of CDC method by Luminex technique in the HLA antibody screening. In a clinical setting, antibodies positive by both methods (MFI values above cutoff) can predict positive CDC crossmatch result with, consequently, refusal of the kidney offer and prevention of the futile organ shipment in the recipient centre. In the case of antibodies that have MFI below the established cutoff and that are likely to be detected by Luminex only, we reassess criteria and parameters beside MFI that should be considered in the accurate and reliable identification of clinically relevant unacceptable HLA antigen mismatches (UAM).

Material and methods

This study is a retrospective analysis of HLA antibody screening results performed at the Tissue Typing Laboratory (TTL), Clinical Institute for Transfusion Medicine, Clinical Hospital Center (CHC) Rijeka, Croatia, from January 2012 to July 2019. Out of 1,729 pre-transplant sera, 1,665 native sera tested in parallel by two methods (CDC and Luminex) were analysed. In the study were not included 64 sera, as 54 were tested partially, by CDC or Luminex method only, and 10 were positive in patients not exposed to any immunizing event. Positivity was detected only by Luminex as an isolated finding and interpreted as false positive. Data were obtained from TTL and CHC documentation related to HLA antibody screening including worksheets, final interpretations of test results and informations about patients' clinical status and exposure to immunizing events.

In the TTL Rijeka, HLA antibody screening is performed quarterly at regular intervals, by the two methods in parallel-CDC and Luminex. Serum collected two to four weeks after the immunization event are also included in the regular screening scheme. CDC method is a three-step assay in which donor lymphocytes, with specific HLA antigens expressed on their surface, are incubated with patient serum. If recipient has donor-specific antibody, it will bind to the complementary antigen, forming an immunocomplex. By adding rabbit serum as a source of complement, the activation of complement components leads to formation of a membrane attack complex (MAC) that damages cell membrane. Vital dyes are used for visualization of dead lymphocyte cells making it visible under the microscope. The degree of lysed cells is expressed as the percentage of panel reactive antibody (%PRA) [11,12]. In our labo-

ratory HLA antibody screening by CDC method is performed using unseparated T+B lymphocytes consisting of a panel of 50 cells, with and without dithiothreitol (DTT) addition in order to distinguish IgG from IgM antibodies. A serum with PRA>5% is considered positive. Along to CDC method, HLA antibody detection is performed with Luminex technology that is based on the principles of flow cytometry using polystyrene microspheres (instead of cells) to which purified glycoproteins (antigens) HLA Class I and/or II are conjugated [15]. The basic principle of this method is that after incubation of beads with the patient's serum, the eventually present IgG antibodies bind to complementary bead-conjugated HLA antigens while the unbound antibodies are washed. Bound HLA antibodies are detected by use of a phycoerythrin (PE) labelled anti-human IgG antibody. Polystyrene microspheres contain two fluorochromes in different ratios making each set of beads unique in their spectral signature, allowing simultaneous detection of up to 100 different sets of beads. The Luminex fluorocytometer uses a system of two lasers-green (wavelength 532 nm) and red (wavelength 650 nm). After measuring signals by detectors, results are processed in a computer program [13]. The degree of fluorescence is expressed as mean fluorescence intensity. MFI value greater than 1,000 is considered as a positivity cut-off. HLA antibody screening by Luminex technology in the TTL Rijeka is performed at two levels according to the guidelines of test manufacturer-Immucor GTI Diagnostics, Inc. (Waukesha, WI, USA; formerly: GenProbe). The first phase is a Lifecodes LifeScreen Deluxe (LMX) assay. It is a qualitative assay and the results are expressed as positive or negative depending on the presence or absence of HLA antibodies. In the case of a positive result, testing is continued with Lifecodes Single Antigen (LSA) I and/or II assays [1,14]. Once specificities have been established, SAB tests are performed once a year or after immunization event. Testing is carried out on a LABScan 200 Flow Analyzer (Luminex, Austin TX, USA). The results are analysed using the computer program Lifecodes® MatchIt software manufactured by Immucor GTI Diagnostics, Inc. (Waukesha, WI, USA). All analysed sera were divided into four groups according to test results: CDC+LUM+ (positive test result by CDC and Luminex); CDC-LUM+ (negative test result by CDC and positive result by Luminex technique); CDC+LUM- (positive result only by CDC method and negative test result by Luminex) and CDC-LUM- (negative test result by both methods).

Furthermore, the results of 174 sera tested by CDC method and Lifecodes Single Antigen I (LSA I) kit were analysed and then compared between CDC+LUM+ and CDC-LUM+ groups. This comparison was limited by several factors. Firstly, CDC screening was performed with unseparated T+B lymphocytes so HLA Class II antibodies could not be detected by this method. In CDC positive sera, HLA-A and HLA-B antibody spe-

cificities were identified, however, specificities of antibodies against HLA-C molecules were difficult to interpret. Thus, comparing the results obtained by CDC and Luminex method for Class II and HLA-C antibodies would not be plausible given the aim of this study. Secondly, in the CDC+LUM- group HLA IgG antibodies were not confirmed by Luminex, so sera from groups CDC+LUM- and CDC-LUM- were not further analysed.

Statistical analysis

Frequency differences with respect to the method and HLA antibody screening results were calculated by the Hi-square test (χ^2). If χ^2 was significant, differences between the groups were checked by T-test for proportions. Numerical values are presented with median, 5th and 95th percentiles and range, as data were not distributed normally. The medians of maximum MFI values between the CDC+LUM+ and CDC-LUM+ groups were compared using the Mann-Whitney U test. The results are presented graphically by box and whisker diagrams.

Cutoff MFI values for the HLA Class I antibodies detected by CDC method and Luminex technique were determined by using receiver-operating characteristics (ROC) curve and cutoff analysis. In the range of limit value, antibodies having MFIs above cutoff will be detected by CDC method and Luminex, while antibodies with MFI values below cutoff will be detected by Luminex technique only. Statistical analyses were performed using MedCalc® v18.2.1 (©1993-2017, MedCalc Software bvba, Ostend, Belgium). All results with the level of $P \leq 0.05$ were considered statistically relevant.

Results

During the study period, 1,665 sera of 296 patients awaiting kidney transplantation were screened for HLA antibody presence by CDC method and Luminex technique in parallel. Almost half sera were antibody negative ($n=867$; 52.1%). Among positive sera, significantly more antibodies were detected by Luminex technique than by CDC method ($\chi^2=58.95$; $P < 0.001$) (Table 1).

Table 1. Results of HLA antibody screening performed by the CDC method and Luminex technique

Methods of HLA antibody screening	LUM - N (%)	LUM + N (%)	Total N (%)
CDC -	867 (52.1)	467 (28.0)	1,334 (80.1)
CDC +	58 (3.5)	273 (16.4)	331 (19.9)
Total	925 (55.6)	740 (44.4)	1,665 (100)

Abbreviations: CDC, complement-dependent cytotoxicity; LUM, Luminex technique

The presence of antibodies against HLA Class I and/or II antibodies were analysed and compared between CDC+LUM+ and CDC-LUM+ groups. HLA Class I antibodies were only detected in twice as less sera as Class II antibodies (16.1% and 34.4%, respectively), while Class I/II antibodies were detected in almost half sera (49.5%). In total, a slightly more HLA Class II than Class I antibodies (56.1% vs. 43.9%) were detected in the pre-transplant patients' sera.

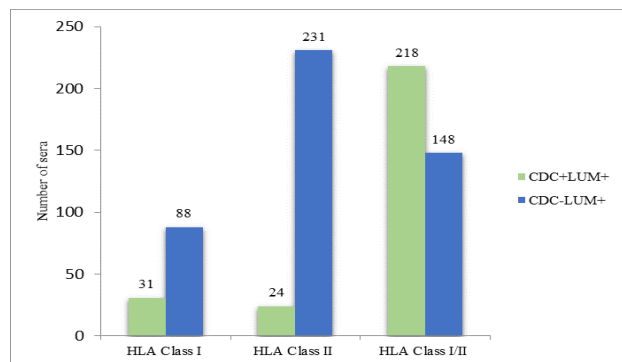


Fig. 1. Distribution of patients' sera according to the HLA Class I and/or II antibody specificity. Abbreviations: CDC, complement-dependent cytotoxicity; LUM, Luminex technique

In the CDC+LUM+ group, most sera ($N=218$; 79.9%) contained HLA Class I and II antibodies while in the CDC-LUM+ group, most sera ($N=231$; 49.5%) gave a positive result for HLA Class II antibodies only (Figure 1).

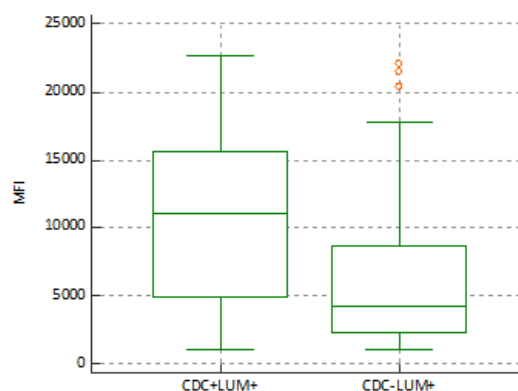


Fig.2. Comparison of median and interquartile range for the highest MFI values from SAB assays for HLA Class I antibodies between CDC+LUM+ and CDC-LUM+ groups (the plot represent from bottom: minimum, first quartile, median value, third quartile, maximum value and outliers). Abbreviations: CDC, complement-dependent cytotoxicity; LUM, Luminex technique; MFI, mean fluorescence intensity; SAB, single antigen bead

For Class I antibodies, median of the highest MFI values between groups CDC+LUM+ and CDC-LUM+ showed statistically higher MFI value in CDC+LUM+ group (median=11,022) than in CDC-LUM+ group (median=4,293), ($P < 0.001$) (Figure 2).

Medians of the highest MFI values from antibodies against HLA-A, HLA-B and HLA-C molecules were compared. While the results for HLA-A and HLA-B antibodies were similar, almost twice as low was median of MFI for HLA-C antibody specificities (Table 2).

Table 3. shows the evaluation of pre-transplant HLA antibody MFI values separately by HLA-A and HLA-B loci in CDC+LUM+ and CDC-LUM+ groups. MFI

Table 2. Medians of the highest MFI values for antibodies against HLA-A, HLA-B, and HLA-C molecules

HLA locus	Median	Range (min-max)	(5 th – 95 th percentile)
A	8.559	1.008-22.215	1.493-20.488
B	9.402	1.033-22.493	1.260-20.621
C	4.018	1.138-22.611	1.317-18.597

Abbreviation: MFI, mean fluorescence intensity

values of both, HLA-A and HLA-B antibodies were statistically higher in group of sera with antibodies detected by CDC and Luminex methods in comparison to the sera in which antibodies were detected by Luminex only. In the CDC-LUM+ group, MFI values of antibodies against

Table 3. Comparison of the median MFI values for HLA-A, HLA-B and HLA-C antibody specificities identified in pre-transplant sera between CDC+LUM+ and CDC-LUM+ groups

Locus	N	CDC+LUM+ MFI			N	CDC-LUM+ MFI			Mann-Whitney U test	
		Median	Range (min-max)	(5 th -95 th percentile)		Median	Range (min-max)	(5 th – 95 th percentile)	U	P
A	79	11.412	1.704-22.215	1.954-21.046	58	4.122	1.008-21.477	1.325-3.975	906	<0.001
B	79	12.802	1.078-22.493	1.453-21.429	62	5.927	1.033-22.057	1.167-7.678	1.433	<0.001

Abbreviations: CDC, complement-dependent cytotoxicity; LUM, Luminex technique; MFI, mean fluorescence intensity

HLA-A*24:02, A*03:01, A*36:01, A*32:01, A*02:03 and HLA-B*07:02, B*27:05, B*27:08, B*07:02, B*08:01, B*81:01, B*56:01, B*44:03, B*37:01 were higher than the median MFI value of antibodies of respective locus in the CDC+LUM+ group (listed in Table 3.).

Cutoff MFI value for HLA antibodies Class I was determined by the Receiver operating characteristic (ROC) curves that represent the graphical relationship between sensitivity and specificity of Luminex technique versus CDC method. Antibodies having MFIs above cutoff will be detected by CDC method and Luminex, while antibodies with MFI values below cutoff will be detected by Luminex technique only. For HLA Class I antibodies cutoff MFI value is 9,204. Area under the ROC curve is 0.720 ($P < 0.001$).

Discussion

HLA antibody screening and identification is an important part of an immunogenetic assessment of potential organ transplant recipients and also one of the most challenging procedures in the TTL. Low sensitivity of the CDC method in HLA antibody detection encouraged development of new techniques and methods among which microbead based platforms such as Luminex, emerged as the most sensitive ones [15]. Higher sensitivity of Luminex technology in HLA antibody detection reported by many researches was also confirmed by this study [10,16,17]. Presence of antibodies in significantly higher number of sera was demonstrated

by Luminex technique than by CDC method (44.4% vs. 19.9%; $P < 0.001$).

Based on the method used, the prevalence of HLA Class I/II antibodies between two groups of sera (CDC+LUM+ and CDC-LUM+) was compared. In the CDC+LUM+ group, antibodies were mostly HLA Class I and II specificities, while in the CDC-LUM+ group most sera contained HLA Class II antibodies. HLA Class I antibodies react with B and T lymphocytes while HLA Class II antibodies react with B cells expressing Class II molecules. Without T and B lymphocyte separation, HLA Class II antibodies cannot be entirely detected by the CDC, so the result of their highest proportion in sera with antibodies detected by Luminex only (CDC-LUM+) was as expected (90.6%). Elegant detection of HLA Class II antibodies which in CDC assay require lymphocyte separation, is one of the great advantages of Luminex technique. Clinically, the highest risk of organ rejection and graft loss have patients with both HLA Class I and II antibodies, which are in this study mostly present in the CDC+LUM+ group [18,19].

The output of Luminex assay is a semiquantitative measure referred as the mean fluorescence intensity (MFI) [12]. According to the MFI values, Class I antibodies detected by CDC and Luminex methods have a significantly higher MFI value than HLA antibodies proven by the Luminex technique alone ($P < 0.001$) which has also confirmed higher sensitivity of Luminex technique. Analysing each HLA Class I locus separately, median MFI values of HLA-A and HLA-B antibodies are twice as high as MFI for HLA-C locus. Peripheral blood lym-

phocytes express HLA-A and HLA-B proteins at similar levels, which are several times higher than HLA-C molecule expression [20]. HLA-C locus has relatively reduced diversity with regard to HLA-A and HLA-B loci [21]. Therefore, the mentioned differences in MFI values, so as difficult interpretation in CDC assay, may be explained by the influence of cell surface expression and polymorphism level of HLA molecules on the occurrence and strength of the antibody.

According to literature, antibodies with MFI value above 7.000-8.000 will give a positive reaction in CDC assay [22,23]. In this study, MFI cutoff value for HLA Class I antibodies was above 9.204, which is consistent with literature data. The median MFI value for HLA-A was 8.559, for HLA-B 9.402. The highest MFI median value was determined for HLA-B antibody specificities as the HLA-B locus is the most polymorphic, it has over 3.000 allelic variants, and the highest number of epitopes are shared by HLA-B locus antigens in comparison to the HLA-A (or HLA-C antigens) [24-26].

The antibody-reactivity pattern against epitope is the basic setting of determining HLA matching at the epitope-level which became a recent topic in organ transplantation. If the recipient and donor share the same epitopes, no HLA antibody will be generated. Although each HLA molecule has a unique set of epitopes, some of them may be present on multiple antigens of different specificities [27]. Today, there are computer programs that determine epitope-level matching identifying mismatched HLA molecules that will not lead to the generation of antibodies in the organ recipient (HLA Matchmaker, Predicted Indirectly ReCognizable HLA Epitopes; PIRCHE, etc.), thereby achieving better match between recipient and organ donor [28]. Although the concept is promising, there are some issues to be addressed before its integration into organ allocation programs. The most important is a system for defining a complete epitope spectrum of all HLA antigens and determination of the biological significance as well as immunogenicity of all eplets [29].

One of the advantages of implementation of Luminex technique into patients' screening at the TTL Rijeka is the increase in organ allocation of highly immunized patients (PRA>85%), while UAMs in such patients are difficult and sometimes impossible to characterize by CDC technique. Also, given the limitation of CDC method in detection of HLA Class II antibodies, the use of Luminex technique has made a significant shift in the identification of lymphocytotoxic HLA antibodies, which ultimately resulted in better immune matching when selecting organs for transplantation. Development of Luminex technology and SAB assays enabled implementation of the "virtual crossmatch" which allows exclusion of donors with UAM based on HLA antibody specificities defined by solid phase assay [30]. This reduces the time of cold ischemia, which in cadaveric

transplantation is one of the most important factors affecting graft survival [31].

The higher sensitivity of Luminex technique in detection of HLA antibodies is of great advantage on the one hand, but it raises many questions and concerns on the other. The strong limitation of Luminex technique is certainly the impossibility to standardize the universal cutoff MFI value that would ensure the detection of only clinically relevant HLA antibodies in the patient's serum. The most common MFI value taken as a positivity cutoff in the literature is MFI>1.000 (the value also used in this study), although each laboratory sets its own limit values based on their laboratory and clinical results [12,32]. MFI values depend on the amount (titre) of antibody, affinity for the antigen, antigen density on the beads that varies within same assay and between manufacturers, non-specific binding of serum components to microspheres and on technical performance of the test, which varies between laboratories and operators [17,21]. Artificially antigen binding to a bead may cause conformational change of protein leading to formation of denatured HLA molecules. The result may be exposure of cryptic epitopes which are normally inaccessible to an antibody, formation of neoepitopes or concealment of immunologically relevant epitopes [17]. In this study, in sera without panel reactive antibodies, some antibody specificities expressed high MFI. This finding raised the question of whether these antibodies were complementing binding? If not, what role do they have in transplant outcome given the high MFI value? Recent modifications of SAB assays allow detection of HLA antibodies that have the capacity to bind C1q or C3d as indicators of complement activation. Published studies are inconsistent regarding clinical relevance of complement fixing characteristics of IgG DSA detected by SAB [4,19,33,34].

The role of HLA antibodies detected by Luminex technique only in the graft rejection has not yet been fully elucidated. Although some studies have shown their harmfulness in terms of increased risk of rejection or adverse effect on graft survival, other studies have failed to confirm this [35-37]. Thus, HLA antibodies detected exclusively by Luminex technique (without confirmation by CDC method) are more considered to be a relative rather than an absolute contraindication for transplantation [4,24,38]. The experience of our laboratory in patients' serum screening with Luminex technique is relatively short. In a study that monitored the outcome of 109 kidney transplants performed at the CHC Rijeka from 2012 to the end of 2015, no significant effect of HLA antibodies detected by Luminex method on one-year graft survival was demonstrated (unpublished observation). Also, of all transplants performed at the CHC Rijeka in the period followed by this study, only three recipients were transplanted across donor specific antibodies. Therefore, views on the

clinical relevance of antibodies detected by Luminex technique in our center are largely based on literature data. Although the group of CDC+LUM- sera were not analysed, these results (found in 3.5% of sera) may have an important impact on final transplant decision giving the possibility of positive crossmatch with a potential donor. Possible reasons may be false results (prozone effect, technical reasons), IgM antibodies, non-HLA antibodies, previous treatment by rituximab or antithymocyte globulin, etc. Some of those doubts can be resolved only by comparing the results of Luminex and CDC assays.

There are some limitations of this study. Most references cited for possible MFI cutoffs present results produced by kits from another manufacturer. Comparative analysis showed they have a similar, but nonidentical, ability to detect HLA antibodies [39]. Over the tested period a variety of lots have been used for Luminex testing so the possible variations in MFI values due to new lots were not considered. False negative Luminex results due to potential interaction of complement with IgG HLA antibodies as the prozone effect could not therefore be avoided in some cases as serum pretreatment or dilution were not performed.

Conclusions

Implementation of advanced technologies brought new insights in the patients' immunization status and the influence of HLA antibodies on organ transplantation outcome. Today, Luminex technology is the most sensitive SPA in HLA antibody detection, accompanied by numerous advantages, but not without challenges that need to be overcome, most notably regarding test performance and data interpretation. MFI values are often used as quantitative assessment of antibody strength and used to monitor patients' clinical status. Though it is a useful tool, there are many more factors to consider in test interpretation. Consequently, MFI values of HLA antibodies represent just a tip of an iceberg and we can partially rely on it. Taking the benefits and limitations of Luminex technique into consideration, according to our results, determination of clinically relevant antibodies pre-, as well as post-transplantation cannot be based on SPA only. Sensitive Luminex technique must be combined with CDC method and the final interpretation of results needs to be based on patient's immunological history and clinical status requiring close collaboration between clinicians and tissue typers.

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Conflict of interest statement. None declared.

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