RESEARCH ARTICLE

False negative single antigen bead assay: Is it always an effect of prozone?

Dharmendra Jain¹ | Jui Choudhuri¹ | Rajni Chauhan¹ | Pranav Dorwal¹ | Deepak Sharma² | Aseem K. Tiwari¹ | Vimarsh Raina²

¹Molecular Genetics Laboratory, Department of Pathology and Laboratory Medicine, Medanta-The Medicity, Gurgaon, Haryana, India

²Chimera Transplant Research Foundation, Masjid Moth, New Delhi, India

Correspondence

Dharmendra Jain, Molecular Genetics Laboratory, Medanta -The Medicity, Gurgaon, Haryana, India.
Email: jain_dharmendra2004@hotmail.com

Background: Bead based flow cytometry and Luminex play a major role in identification of alloantibodies in renal transplant work-up. Strong sensitization events may lead to prozone phenomenon that can affect single antigen bead (SAB) assay and result in false negativity. However, this can also be due to high titer of other blocking antibodies. While methods like, heat inactivation, C1 inhibitor, Ethylene diamine tetra-acetic acid and Dithio threitol treatment can remove interfering antibodies of complement and IgM, these methods are not optimal if false negativity is due to prozone effect, which is high titer of antibodies alone.

Methods: We hereby present a case of a highly sensitized renal transplant recipient with 64% panel reactive antibody positivity (PRA) and a subsequent negative SAB assay. This paradoxical finding hinted at SAB being a false negative result and serial dilutions were used to perform further tests.

Results: Serum dilutions lead to positive flow based panel reactive antibody (PRA) and flow cytometry crossmatch (FCXM), with an increasing trend in FCXM.

Conclusions: In highly sensitized patients serial dilution should be considered during a transplant work-up to avoid missing any underlying antibodies. Serum dilution can be used as first option to circumvent prozone. Also, interference of other antibodies should not be labeled as prozone effect.

KEYWORDS

human leukocyte antigen, panel reactive antibodies, prozone effect, renal transplant, single antigen bead assay

1 | INTRODUCTION

Identification of prozone antibodies is a crucial part of pre-renal transplant work-up. Though complement dependent cytotoxicity (CDC) was the conventional method for over 40 years, in recent times more sensitive assays such as bead based flow cytometry and Luminex have come to play a major role. In these assays, commercially available beads are pre-coated with either recombinant or native cell lined HLA antigens. When incubated with patient serum, the antibodies bind to these antigens, which are subsequently detected by secondary antibodies tagged with fluorochrome.

The bead based assays have multiple advantages over the conventional test. These include; firstly, the test can be performed on a stored sample and even done retrospectively as fresh donor cells are not needed. Also the beads cover a wider range of HLA antigens and help in identifying particular donor specific antibodies which subsequently used for donor selection by mean of performing virtual crossmatch.¹ In fact Luminex based solid phase assay has been proven to be more sensitive and specific for identification of donor specific antibodies than other technologies such flow cytometry.² Combination of these assays are now extensively used in pre-transplant work-up, however in rare cases like the one reported here, a further understanding of the

concept of prozone phenomena is needed to interpret the test result correctly.³

Conventionally, prozone phenomenon is defined as a false negative response due to high antibody titer which interferes with formation of antigen- antibody complex.⁴ However, there is literature suggesting that false negative response may be due to high titers of IgG antibodies or other causes such as interference of IgM antibodies (which are otherwise not clinically significant) or excess of C1q antibodies which hamper the binding of the labeled anti- IgG antibody used as a read-out in bead based flow cytometry and Luminex assay.⁵ However, the latter is frequently confused with prozone effect.

Here, we describe a case where results of bead based assays were compromised due to prozone effect and this was further established and proven to be due to high titer of anti-HLA IgG antibodies alone.

2 | CASE REPORT

We present the case of a 37 year old male who was being worked up for a third renal transplant. The patient had a history of hyper acute allograft rejection during first renal transplant with his mother as the donor and chronic rejection during post second transplant with his mother-in law as the donor. Both the renal grafts were removed post rejection. A Panel Reactive antibody (PRA) by flow cytometry was done in our laboratory and showed 64% Class II positivity.

He was continued on hemodialysis (MHD) and referred back for further work-up after 4 months for assessment for a third prospective renal transplant. During these last four months, he was not on immunosuppression and there was no new sensitizing event. In view of a previous positive PRA result, a single antigen bead (SAB) assay was performed to identify the specificity of the antibodies for a subsequent virtual crossmatch with the prospective donor and to identify donor specific antibodies (DSA). The qualitative detection for IgG anti-HLA antibodies was done using the Lifecodes LSA Class I & Class II kits, using Luminex[®] (Immucor Transpalnt diagnostics, inc, Stanford, CT, USA). The recipient serum was allowed to incubate with the beads coated with recombinant HLA antigens and after removing the excess serum antibodies by wash buffer, an anti-human IgG phycoerythrin conjugate (PE) was added. Acquisition of the beads was done on Luminex using Xponent software. The analysis was done using Xponent match IT antibody software. The signal intensity from each bead was compared to the signal intensity of negative control beads included in the bead preparation to determine if the bead was positive or negative for bound alloantibody. The BCM value of more than 1000 was used as cut off for a positive bead. SAB result was negative for Class I and Class II with highest Background corrected MFI (BCM) value of 94 and 747, respectively and the flow crossmatch showed weak positivity for B cells alone.

Since this report varied significantly from the previous result of PRA (64% Class II), we considered this as possibly due to other factors such as- prozone effect and high titers of other blocking antibodies. As a feasible option to overcome this, the serum was diluted and flow PRA was re-run with neat, 1:4, 1:8, 1:16 and 1:32 dilutions.

A commercial PRA screening kit was used (One Lambda Inc., Canoga park, CA, USA). A 20 μ L of serum was mixed with 5 μ L of PRA Class I and Class II beads and incubated for 30 minutes. The beads were then washed at 9000 g for 3 minutes with 100 μ L of 1 $\times$ wash buffer. Added 1 $\times$ 100 μ L of Goat anti-human IgG (Fc γ)-FITC and incubated for 30 minutes. The beads were washed again and suspended in 300 μ L of wash buffer. The beads were acquired at a logarithmic scale on FSC v/s SSC dot plot. The Class I and II beads were then gated on a PE v/s SSC dot plot. On FL1 (FITC) histogram more than 10% of the events for Class I and Class II beyond the negative control were used to call a positive test (Figure 1).

The results of PRA were as follows: Neat serum was negative for both Class I and II, while positivity was seen for Class II in further dilutions of 1:4, 1:8 and 1:16. In 1:4, 1:8 and 1:16 dilution the positivity was 48.17%, 48.41% and 52.63%, respectively for Class II and at 1:32 dilution it became negative (Table 1). Neat serum and all the serum dilutions were negative for HLA Class I.

The trend of increasing positivity with dilutions was reproduced by performing flow cytometry crossmatch (FCXM) which was performed with minor modifications to the protocol described earlier.⁴ A 0.25 million donor's isolated peripheral blood mononuclear cells suspended in 50 μ L of media were mixed with 50 μ L of test serum and incubated for 30 minutes at 4°C. The cells were then washed with cold media. A 50 μ L of Goat-Anti Human IgG (Jackson ImmunoResearch Laboratories inc., West Grove, PA, USA), 10 μ L of Anti-CD22-PE (Beckton Dickinson, Franklin Lakes, NJ, USA) and Anti CD3-PerCp (Beckton Dickinson) were added to the cell pellet and incubated for 30 minutes at 4°C. The cells were then washed and re-suspended in 500 μ L of PBS. The lymphocytes were gated on FSC-SSC dot plot. These gated lymphocytes were then resolved into B and T-cells using CD3 and CD22 dot plot. Each of these populations was gated and the MFI of these was evaluated for IgG FITC. A median channel shift (MCS) of 50 and 80 for T and B cells respectively, beyond the negative control value, were used to call a test positive (Figure 2). The MCS of IgG for B cells were 84, 144, 224, 151 and 67 for neat, 1:4, 1:8 and 1:16 and 1:32 dilutions, respectively (Table 1).

To rule out negative result due to high titers of C1q antibodies, serum was treated with EDTA (1:20 ratio of 6% EDTA).³ Also, In order to rule out the effect of IgM antibodies serum was heat inactivated with incubation at 56°C for 30 minutes. Both the EDTA and heat inactivated serums were processed for PRA and FCXM, however the result continued to remain negative. The increasing trend of positivity with Flow PRA and FCXM (seemingly the prozone effect) were further confirmed by running SAB Class I assay at 1:4 serum dilution which showed the positive results with 18% PRA with highest BCM value of 2359.

3 | DISCUSSION

The present case is of a patient awaiting third renal transplant who's SAB and PRA were negative despite a strong sensitization history and a 64% Class II PRA positivity four months prior. A further detailed work-up showed strong positivity for Class I and II alloantibodies and

FIGURE 1 Representation of Flow-PRA showing. (A) FSC/SSC dot plot showing acquired beads. (B) PE/SSC dot plot showing separation of Class I and II beads. (C) IgG-FITC histogram showing percent PRA of Class I beads. (D) IgG-FITC histogram showing percent PRA of Class II beads

TABLE 1 PRA and flow crossmatch results with different dilutions of serum

Serum dilution	Panel reactive antibody (PRA)		Flow crossmatch (FCXM)			
	Class I (%)	Class II (%)	T cell		B cell	
			MFI	MCS	MFI	MCS
Negative Control	0.26	0.56	67	NA	121	NA
Positive Control	98.64	99.97	553	486	777	656
Neat	0.70	0.28	63	-4	205	84
1: 4	0.04	48.17	41	-26	265	144
1: 8	0.29	48.41	26	-41	345	224
1: 16	0.57	52.63	66	-1	272	151
1:32	0.20	1.93	33	-34	188	67

MFI, Mean fluorescence intensity; MCS, Median channel shift (MFI of test- MFI of negative control); NA, Not applicable.

hence this unexpected initial negative result was hypothesized to be an effect of prozone phenomena.

Prozone effect and complement/IgM interference have been used interchangeably in literature. However, Berth M has described them as separate entities.⁶ While different methods such as, heat inactivation, EDTA and DTT treatment can remove

interfering antibodies of complement and IgM these do not work if the false negative result in an assay is due to prozone effect that is high titers of antibodies alone. The problem of prozone effect can be detected and easily solved in the routine clinical laboratory by diluting the serum or plasma sample. It has been suggested previously that interference of matrix elements or other

FIGURE 2 Representation of Flow cytometry crossmatch showing. (A) FSC/SSC dot plot showing acquired lymphocyte population. (B) PE/PerCp dot plot showing separation of T and B cells. (C) IgG-FITC histogram showing fluorescence intensity of T cells. (D) IgG-FITC histogram showing fluorescence intensity of B cells

non-analyte-related factors in laboratory assays should not be referred to as a prozone effect.⁶

Various suggestions have been put forward by studies to overcome these complement/IgM interference and/or prozone problem. These adaptations include dilution of sera, pre-treatment with DTT, EDTA, C1 inhibitor and heat inactivation.⁷ Dilution of sera has been reported to be useful to overcome the prozone phenomenon.⁸ In the present case, we used dilution as the first step to identify alloantibodies, and results were positive in serial dilutions. This is the primary method when the negative result is due to high titers of antibody alone and is a feasible option in the laboratory.

An insignificant difference in the percent PRA positivity in different dilution can be understood by the fact that the PRA reflects percentage of the positive beads for the HLA antibodies and not the titer of antibodies and therefore once the prozone effect was taken care by the initial dilution of 1:4, subsequent dilution was of no help.⁹ In the FCXM, the titer dependent serial dilution of the sera resulted in the increasing trend of MFI of IgG for B- cells, though it is reduced in subsequent dilution of 1:16. At 1:32 both the PRA and FCXM became negative probably due to dilution of sera resulting in antibody titer below the limit of detection of the assay.

Complement dependent cytotoxicity (CDC) test has always been done with serial dilutions of serum, intended to avoid missing such cases. We wanted to confirm further that this was isolated Prozone or due to interfering complement/IgM antibodies.

IgM antibodies were also considered as a possible reason for unexpected false negative result which act by hindering binding of the tagged secondary IgG antibody. Dithio threitol (DTT) is often used in laboratories to differentiate IgM and IgG antibodies since the former is more commonly auto-antibodies and not a contraindication for transplant. It can also be used to remove these interfering IgM antibodies.⁹ Heat inactivation is also used to remove IgM antibodies from serum by cleaving the disulphide bonds in the pentamer structure of IgM. Heat inactivation and DTT have similar mechanism of action, however the former is less specific.¹⁰ Heat inactivation is recommended as the method in the bead based assay kits. Both these methods were also used in our case, however it continued to yield negative result and ruled out IgM as a factor leading to false negative results.

Antibodies to C1q have also been described in similar cases. Excessive antibodies to C1q can block the binding sites the primary IgG antibody, leading to negative results. EDTA acts as a chelating agent and effectively removes calcium ions required for C1q assembly and

blocks complement activation and are considered the best method to overcome the false negative result due to C1q antibodies.³ EDTA treatment of serum was also applied in our case but result continued to remain negative.

Since the case, could only be resolved with dilution of sera and other described methods did not help, we concluded that this was a case of high titers of prozone IgG antibodies alone- a conventional "Prozone effect".

4 | CONCLUSION

We conclude that serum dilution can be used as a first option while testing highly sensitized patients, to avoid missing any underlying antibodies. Being an easy and feasible option it can be included in all platforms of testing to circumvent both high antibody titers and interference of complement/IgM antibodies which may lead to false negative results. Prozone effect and interfering antibodies should be considered as separate phenomenon since treatment with heat and EDTA can help overcome blocking antibodies, but cannot help in case of prozone ie high IgG antibodies titers alone.

REFERENCES

1. Lachmann N, Todorova K, Schulze H, Schönemann C, Luminex® and its applications for solid organ transplantation, hematopoietic stem cell transplantation, and transfusion. *Transfus Med Hemother*. 2013;40:182-189.
2. Muro M, Llorente S, Marín L, et al. Acute vascular rejection mediated by HLA antibodies in a cadaveric kidney recipient: discrepancies between Flow PRA, ELISA and CDC vs luminex screening. *Nephrol Dial Transplant*. 2005;20:223-226.
3. Schnaidt M, Weinstock C, Jurisic M, Schmid-Horch B, Ender A, Wernet D. HLA antibody specification using single-antigen beads-a technical solution for the prozone effect. *Transplantation*. 2011;92:510-515.
4. Schiettecatte J, Anckaert E, Smits J. Interferences in immunoassays. In: Norman HL, Christopoulos TK, eds. *Advances in Immunoassay Technologies*. Rijeka: InTech;2013:45-62.
5. Haarberg KM, Tambur AR. Detection of donor-specific antibodies in kidney transplantation. *Br Med Bull*. 2014;110:23-34.
6. Berth M. Complement interference is not the same as a prozone phenomenon. *Am J Transplant*. 2016;16:1638.
7. Weinstock C, Schnaidt M. The complement-mediated prozone effect in the Luminex single-antigen bead assay and its impact on HLA antibody determination in patient sera. *Int J Immunogenet*. 2013;40:171-177.
8. Jacobs JF, van der Molen RG, Bossuyt X, Damoiseaux J. Antigen excess in modern immunoassays: to anticipate on the unexpected. *Autoimmun Rev*. 2015;14:160-167.
9. Kılıçaslan Ayna T, Özkızılcık Koçyiğit A. Detection of prozone antibodies by flow cytometer, flow cytometry-select topics, M.Sc.; 2016. Schmid I, ed. InTech. <https://doi.org/10.5772/62553>. Available from: <https://www.intechopen.com/books/flow-cytometry-select-topics/detection-of-anti-hla-antibodies-by-flow-cytometer>. Accessed October 26, 2016.
10. Al-Muzairi IA, Mansour M, Almajed L, Alkanderi N, Alshatti N, Samhan M. Heat inactivation can differentiate between IgG and IgM antibodies in the pretransplant cross match. *Transplant Proc*. 2008;40:2198-2199.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Jain D, Choudhuri J, Chauhan R, et al. False negative single antigen bead assay: Is it always an effect of prozone? *J Clin Lab Anal*. 2018;32:e22237. <https://doi.org/10.1002/jcla.22237>