

False negative results in luminex panel reactive antibody identification assay: Should we be alert on them?

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Abstract

Aim: Panel Reactive Antibodies (PRA) analysis is important not only in virtual cross-match of donors and recipients but also in follow-up of recipients immune response after transplantation. Although bead based tests give faster and more reliable, it may have false positive and negative results. We investigated HLA groups of patients with false negative results and the frequencies of anti-HLA antibodies in all three false negative situations.

Materials and Methods: PRA results of all patients were divided into 3 groups according to negative controls and samples results. First one is negative controls were false negative and samples were false negative (1st Situation); second one is negative controls were false negative but samples were true negative (2nd Situation); third one is negative controls were true negative but samples were false negative (3rd Situation). All serum samples were tested by bead based Luminex assay.

Results: While anti-HLA-A68, A24, A32 were the most frequent antibodies for the first situation, anti-HLA-A03,24,32 were the most frequent three antibodies for the second situation. In case of anti-HLA-B antibodies; anti-HLA-B38, B44, B48 were detected for the first situation, and anti-HLA-B44, B58, B49 were detected for the second situation. In case of class II antibodies; we detected anti-HLA-DRB₁10, DRB₁15 and DRB₁51 antibodies as the most frequent three antibodies at 1st situation. Frequencies of anti-HLA antibodies of 120 patients with false negative results were determined in serum samples. In case of 3rd situation, while anti-A23, A68, A24; anti-B06, B04, B39 and anti-Cw07 were the most frequent anti-HLA Class I antibodies, anti-HLA DQ07, DQ05, DQ04 and anti-HLA-DRB₁52, DRB₁13, DRB₁53 were the most frequent HLA-Class II antibodies detected in serum samples.

Conclusion: Samples with false negative PRA results should be repeated and these samples should be recorded in the laboratory for internal control system.

Keywords: Bead Assay; HLA; PRA analysis

INTRODUCTION

The importance of antibodies specific to human leukocyte antigens (HLA) Class I and Class II in tissue and organ transplantation has been known for over 40 years (1-4). These antibodies, also called as panel reactive antibodies (PRA), can be developed by various sensitizing events such as blood transfusion, pregnancy and organ transplantation (1,5-7). These sensitized patients waiting for renal transplantation are at risk for hyperacute rejection, acute rejection, antibody-mediated rejection, delayed graft function, and longer-term complications that may develop after transplantation (8-10). Therefore, the presence and specificity of anti-HLA antibodies have been determined in patients waiting for renal transplantation, since 1960s (2,8,9). These tests are known as PRA tests and have two different applications known as screening and identification. PRA screening tests indicate the

presence or absence of class-I and class-II HLA antibodies in patient's serum samples. Patients with negative PRA class-I/class-II screening results are considered as non-sensitized; whereas for patients with positive results, an additional test is performed named as PRA class I/II identification tests to determine specificity of allo-IgG antibodies (11,12). The quantitative value found in the identification test is used to determine the alloantibody specificity and antibody panels to predict the result of virtual cross-match with the donor and also to decide the priority of patients for transplantation.

PRA identification test can be performed by flow-cytometry, ELISA and Luminex. It has been reported that Luminex method gives faster and more reliable results in different studies. However, as in other methods, this method may also have false positive and negative results (13-15). That may appear not only in samples but also in

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controls. Since the controls show if the test work correctly, false positive or negative results in controls should also be considered in attention. Two types of errors could appear in that solid phase assay. First one is false positive result, which is caused by denatured HLA antigens and incongruity on cut-off values. The second one is false negative results which is caused either by interaction of complement with IgG or prozone effect. The latter one may appear in three ways; the first one appears as negative control is false negative and sample is false negative; second one appears as negative control is false negative but sample is true negative; third one is negative control is true negative but sample is false negative. In the literature, there are less amount of knowledge about the properties of those patients in three different PRA situations. In that manuscript, we investigated HLA groups of patients with false negative results and the frequencies of anti-HLA antibodies in all three false negative situations and discuss the importance of these results in PRA analysis.

MATERIALS and METHODS

The work has been conducted according to the principles expressed by Declaration of Helsinki and approved by the Ethics Committee of Inonu University with approval number 2019/4-6.

Patients and Samples

A total of 120 patients (between 2015 and 2016) who were admitted to Inonu University Faculty of Medicine, Liver Transplantation Institute were included in this study. Blood samples were collected from 60 male and 60 female patients who did not receive any desensitization treatment with Rituximab, intravenous immunoglobulins and plasmapheresis before liver transplantation to identify Panel reactive antibodies against Class-I and Class-II HLA. PRA identification test was performed by using Luminex method.

PRA results of all 120 patients were false negative and divided into 3 groups according to negative controls and samples results. First one is grouped as negative controls were false negative and samples were false negative (named as 1st Situation); second one is negative controls were false negative but samples were true negative (named as 2nd Situation); third one is negative controls were true negative but samples were false negative (named as 3rd Situation).

PRA Analysis

All serum samples were tested on Luminex with the reagents from the Luminex PRA-identification kit (LIFECODES Class I / II ID; GEN-PROBE). The samples were studied following the method as described before (16). Briefly, 12.5 µl. serum samples of each patient and 5 µl. Luminex beads coated with class I / II antigens, were added to relevant wells in 96 well plates and incubated for 30 minutes. At the end of incubation period, 96 wells plates washed 3 times by using the vacuum manifold system. Following that, 50 µl of anti-human phycoerythrin-IgG

conjugate was added to the wells and incubated for 30 min. All incubations were carried out at room temperature, in the dark and by using a rotator system. At the end of the period, the plate was loaded onto the Luminex-Life-Match and the results were analyzed in the Quick-Type program. The signal intensity emitted from each bead was compared with the beads treated with the negative and positive control serum samples determined by the manufacturer. Luminex PRA class I or II test results were used to determine the specificity and percentage of HLA class I and class II antibodies.

Statistical Analysis

Frequency analysis of HLA groups and HLA antibodies were determined by using by using Statistical Programme for Social Sciences (SPSS) 10.0 statistical software.

RESULTS

We analyzed HLA groups of patients with false negative results to determine the frequencies of HLA-A, HLA-B and HLA-DRB1 in liver transplant patients (Table 1). Most frequent HLA-A alleles were HLA-A*01 and HLA-A*02 for three situations. HLA-B*51 was the most frequent HLA-B allele which was observed for 3rd situations. Additionally; HLA-DRB₁*11 was frequent for 3rd situations. Besides that; HLA-DRB₁*04, HLA-DRB₁*03, HLA-DRB₁*15, HLA-DRB₁*07, HLA-DRB₁*08 were the frequent HLA-DRB₁ alleles for three situations.

Table 1. Frequencies of HLA-A, HLA-B and HLA-DRB₁ alleles of patients in three situations of false negative results.

Groups	Frequency of HLA-A	Frequency of HLA-B	Frequency of HLA-DRB ₁
1 st Situation (7 patients)	A*01 (14.3%)	B*51 (14.3%)	DRB ₁ *04 (28.6%)
	A*02 (14.3%)	B*49 (14.3%)	DRB ₁ *11 (21.4%)
	A*26 (14.3%)	B*20 (7.1%)	DRB ₁ *01 (14.3%)
2 nd Situation (9 Patients)	A*01 (14.3%)	B*18 (14.3%)	DRB ₁ *07 (14.3%)
	A*02 (14.3%)	B*37 (14.3%)	DRB ₁ *08 (14.3%)
	A*23 (14.3%)	B*44 (14.3%)	DRB ₁ *15 (7.1%)
3 rd Situation (104 Patients)	A*02 (38.1%)	B*51 (30.2%)	DRB ₁ *11 (31.7%)
	A*01 (22.2%)	B*35 (20.6%)	DRB ₁ *03 (23.8%)
	A*24 (17.5%)	B*08 (9.5%)	DRB ₁ *15 (23.8%)

Principally antibody levels should not be determined in negative controls. However, anti-HLA antibodies have been detected in negative controls of some studies. We analyzed frequencies of anti-HLA antibodies detected in negative control samples for first two situations (Table 2). While Anti-HLA-A68, A24, A32 were the most frequent antibodies for the first situation, Anti-HLA-A03,24,32 were the most frequent three antibodies for the second situation. In case of Anti-HLA-B antibodies; anti-HLA-B38, B44, B48 were detected for the first situation, and anti-HLA-B44, B58, B49 were detected for the second situation.

In case of class II antibodies; we detected anti-HLA-DRB₁10, DRB₁15 and DRB₁51 antibodies as the most frequent three antibodies at 1st situation. Interestingly; we did not detect anti-HLADRB₁ and anti-HLA-DQ antibodies at 2nd situation.

Frequencies of anti-HLA antibodies of 120 patients with false negative results were determined in serum samples (Table 3). Anti-A23, -A66 and -B08, -B39, -B44, -Cw07 were the most frequent anti-HLA antibodies detected

in serum samples for the 1st situation. Anti-HLA-DQ5, DQ7 and DQ4 antibodies were at highest frequency in comparison to anti-HLADRB₁ antibodies. In the second situation, the anti-HLA antibody could not be detected as true negative results were obtained in the analyzes. In case of 3rd situation, while Anti-A23, A68, A24; Anti-B06, B04, B39 and Anti-Cw07 were the most frequent anti-HLA Class1 antibodies, Anti-HLA DQ07, DQ05, DQ04 and Anti-HLA-DRB₁52, DRB₁13, DRB₁53 were the most frequent HLA-Class-II antibodies detected in serum samples.

Table 2. Analysis of antibodies detected in false negative controls. Frequencies of Anti-HLA antibodies determined in Negative Controls (NC) for three situations. ND abbreviated as 'Not Determined'

Groups	Frequency of NC α-HLA-A	Frequency of NC α-HLA-B	Frequency of NC α-HLA-Bw	Frequency of NC α-HLA-Cw	Frequency of NC α-HLA-DRB ₁	Frequency of NC α-HLA-DQ
1 st Situation	A68 (14.3%)	B38 (7.1%)	Bw06 (27.3%)	Cw04 (17.6%)	DRB ₁ 10 (17.6%)	DQ05 (33.3%)
	A24 (11.4%)	B44 (7.1%)	Bw04 (9.1%)	Cw07 (17.6%)	DRB ₁ 15 (17.6%)	DQ06 (33.3%)
	A32 (11.4%)	B48 (7.1%)		Cw08 (17.6%)	DRB ₁ 51 (17.6%)	
2 nd Situation	A03 (10.5%)	B44 (7.3%)	Bw04 (%50)	Cw01 (12.7%)	ND.	ND.
	A24 (10.5%)	B58 (7.3%)	Bw06 (%50)	Cw07 (12.7%)		
	A32 (8.4%)	B49 (5.6%)		Cw03 (9.9%)		
3 rd Situation	ND.	ND.	ND.	ND.	ND.	ND.

Table 3. Analysis of antibodies detected in false negative serum samples. Frequencies of Anti-HLA antibodies determined in serum samples with false negative results. ND abbreviated as 'Not Determined'

Groups	Frequency of PRA α-HLA-A	Frequency of PRA α-HLA-B	Frequency of PRA α-HLA-Cw	Frequency of PRA α-HLA-DR	Frequency of PRA α-HLA-DQ
1 st Situation	A23 (20.0%)	B08 (12.5%)	Cw07 (23.1%)	DRB ₁ 13 (10.0%)	DQ05 (25.0%)
	A66 (20.0%)	B39 (12.5%)	Cw12 (15.4%)	DRB ₁ 14 (10.0%)	DQ07 (25.0%)
	A33 (13.3%)	B44 (12.5%)	Cw16 (15.4%)	DRB ₁ 52 (10.0%)	DQ04 (16.7%)
	ND.	ND.	ND.	ND.	ND.
2 nd Situation					
3 rd Situation	A23 (9.7%)	B06 (13.5%)	Cw07 (13.0%)	DRB ₁ 52 (9.8%)	DQ07 (18.6%)
	A68 (8.7%)	B04 (11.9%)	Cw12 (10.7%)	DRB ₁ 13 (8.2%)	DQ05 (17.1%)
	A24 (7.7%)	B39 (5.0%)	Cw03 (9.6%)	DRB ₁ 53 (8.1%)	DQ04 (15.6%)

DISCUSSION

Anti-HLA antibodies are one of the important parameter monitored both before and after transplantations not only at transplantation decision phase but also for patient follow-up (9,17,18). Therefore, on the basis of the specificity of HLA antibodies and the determination of panel reactivity, the chances of success in transplants can be increased by careful screening of patient sera (19).

Different PRA methods are used worldwide to measure the sensitization level and predict prognosis in potential solid organ recipients. It is also stated that Luminex method is more useful and is more advantageous than other PRA methods such as ELISA, flow cytometry and CDC in terms of not requiring a certain expertise in the analysis of the results. Luminex has been proven to be more sensitive than other tests in terms of sensitivity and specificity (16,20,21).

Although many advantages have been proven with the above studies, PRA identification tests studied by luminex method may arise some difficulties especially at control values. In that study we grouped those difficulties according to the results of negative controls and sample values. First one is grouped as negative controls were false negative and samples were false negative (named as 1st Situation); second one is negative controls were false negative but samples were true negative (named as 2nd Situation); third one is negative controls were true negative but samples were false negative (named as 3rd Situation). In case of false negatives in controls, technical errors should be fixed before repeating the test. However, the causes and solutions of false negative samples may not be that simple. As known when the HLA antibody titers are high, a false negative test can occur due to the prozone phenomenon. High titer antibodies cause complement activation and deposition of those proteins on the bead which then prevents HLA antibody binding to the antigen on the beads. Also binding of pentameric IgM antibodies or other serum factors to the beads can arise similar situation. These issues can be overcome either by serum dilution or treatment of serum samples with dithiothreitol. Drugs such as Ivlg can also interfere with the specific binding of HLA antibodies to the HLA antigens on beads. False negative results resulting from the three reasons mentioned above can be easily resolved (15,22). However, there is a fourth reason which is known as epitope sharing; the solution in that may not be as simple as the others. In epitope sharing; distinct HLA antigens on different beads share common antibody binding sites leads to binding of an anti-HLA antibody to more than one bead (23). That causes either reduction in the Mean Fluorescent Intensity (MFI) on a single bead or elevation in the MFI of negative beads. In that situation, which anti-HLA antibodies caused those specious results should be followed regularly.

According to our data; serum samples with false negative results were often detected from individuals with HLA types HLA-A*01, A*02; B*51 and DRB₁*11. PRA identification tests of patients in these HLA types should be analyzed carefully. In the analysis of the negative controls in the PRA identification tests, antibodies against HLA-A24; B44; Bw06, Bw04; Cw07; DRB₁10, DRB₁51, DRB₁15 and DQ05, DQ06 were frequently detected. If the donor HLAs involves one of these allele genes, negative controls of PRA identification test should be carefully analyzed. Antibodies against HLA-A23, B39, Cw07, Cw12, DRB₁13, DRB₁52 and DQ5, DQ7 were frequently detected in the recipient samples with false negative results. If a donor HLA allele gene involves these antigen types it may be useful to study PRA identification tests carefully and to work with appropriate dilutions.

In the literature mostly false positive results were reported. In et al. reported a false positive reaction against HLA Class II molecules detected in Luminex single antigen bead assays. To rule out that problem flow cytometric crossmatches were performed. In samples with moderate

and weak results in Luminex were repeated by B-cell flow cross match test and the results were observed as negative and positive, respectively. They mentioned that sera samples showed an atypical reactivity to denatured HLA-DRB₁*09:01, DRB₃*01:01, *02:02, *03:03 (13). Jacob et al. also reported a positive virtual crossmatch with negative flow crossmatch results in two cases. They determined that identification of antibodies to denatured HLA-B*44:02 by using the LIFECODES LSA Single Antigen test (24).

Jain et reported a case report with false negative data. They overcome that problem by serial dilution and by comparing their data with flow PRA assay. They concluded that, results of bead based assays can be compromised due to prozone effect and this further established and proven to be due to high titer of anti-HLA IgG antibodies alone (14,25).

In our study there are two distinct limitations. One of them is, we do not know donor HLA types thus, the importance of anti-HLA antibodies type in case of virtual cross match would be limited. Second one is we did not work with diluted samples as mentioned above. That is because we realized that false negative data is not only observed in recipient samples but also in test controls.

CONCLUSION

As a result, laboratories should be vigilant about false negative PRA identification results in serum samples and test controls, and should notify the laboratory manager of this rate to avoid the risk of false analysis by recording the frequency of these negative results.

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