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Original Scientific Paper

DETERMINATION OF THE PRA POSITIVITY PERCENTAGE IN MALE PATIENTS WITH CHRONIC KIDNEY DISEASE BY USING FLOW CYTOMETRY TECHNIQUE

Fatma Avcı Merdin[1], Hüseyin Koçak[2] and Sadi Köksoy[3]

[1]Akdeniz University Hospital, Department of Internal Medicine, Antalya, Turkey;
[2]Akdeniz University Hospital, Department of Internal Medicine, Division of Nephrology, Antalya, Turkey;
[3]Akdeniz University Hospital, Department of Immunology, Antalya, Turkey

SUMMARY – The antibodies directed against human leukocyte antigen (HLA) molecules, which play a crucial role in allograft histocompatibility, are called anti-HLA antibodies. Anti-HLA antibodies against foreign HLA molecules may be present in patients with chronic kidney disease even before transplantation. The panel reactive antibody (PRA) test is used to measure the renal transplant candidate's immune sensitivity to HLA molecules other than their own HLA molecules by assessing the diversity of anti-HLA antibodies in the blood of these patients. This study aimed to determine the PRA values and the percentage of PRA positivity of Turkish male patients with chronic kidney disease (CKD), who had not been sensitized by the major known causes (those with no history of organ or tissue transplantation, those with no history of blood transfusion), who had not been diagnosed with any autoimmune diseases, and who had not been under immunosuppressive treatment. The study included 60 male patients aged over 18 years. All of the patients were followed up with a diagnosis of CKD at the Nephrology Clinic, Internal Medicine Department, Akdeniz University Medical Faculty Hospital. None of the patients included in the study was sensitized by a known mechanism previously (they did not have blood transfusion or organ transplantation). Glomerular filtration rate (GFR) levels of all patients were below the level of 60 mL/min/1.73 m^2. Patient data including their age information, etiology of CKD, accompanying diseases, and information about dialysis modalities were recorded. HLA antibody percentage was determined by the flow cytometry technique. Statistical data analysis was performed by using SPSS 22.0 (Statistical Package for Social Sciences, Version 22.0). The values of p less than 0.05 were considered statistically significant. Twenty patients were receiving dialysis treatment due to end-stage renal disease. Of the 60 patients included in the study, 25% showed PRA positivity; 28.3% of all study patients were found to be positive for anti-HLA class I antibodies and 26.7% of all study patients were found to be positive for anti-HLA class II antibodies on separate analysis for anti-HLA class I and anti-HLA class II antibody positivity. When the patients were categorized as PRA negative and PRA positive in two groups, there were no differences between the groups according to mean age, percentage of hemodialysis patients, percentage of peritoneal dialysis patients and presence of accompanying chronic diseases (such as hypertension, type 2 diabetes mellitus, hyperlipidemia, nephrolithiasis, coronary artery disease). In addition to this, evaluation of the GFR levels showed that the PRA positive group contained a significantly higher percentage of end-stage renal disease patients (GFR <15 mL/min/1.73 m^2) as compared with the PRA negative group. Detailed analysis of the percentages of PRA levels in the PRA positive patients, which was carried out to determine the degree of sensitization, showed that the PRA values were over 80% in 11.77% of the patients positive for anti-HLA class I antibodies. On the other hand, PRA values were within the range of 15%-80% in 88.23% of the patients who had anti-HLA class II antibodies. The PRA values were below 80% in all of the patients positive for anti-HLA class II antibodies and those positive for both anti-HLA class I and class II antibodies. In conclusion, PRA levels of the candidates for kidney transplantation should always be measured to assess their state of sensitization before transplantation, even though they have no risk factors known to cause anti-HLA antibody development.

Key words: *Chronic kidney disease; Flow cytometry; Anti-HLA antibodies; Renal transplantation*

Correspondence to: *Fatma Avcı Merdin, MD*, Ankara University School of Medicine, Department of Internal Medicine, Division of Endocrinology and Metabolism, Altındağ, Ankara, Turkey
E-mail: fatma_avci.md@hotmail.com

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Introduction

Chronic kidney disease (CKD) is an important public health problem[1]. Dialysis procedures commonly used in patients with end-stage renal disease can be extremely damaging in both financial and emotional terms. On the other hand, kidney transplantation in these patients affords a better quality and healthier life than do renal replacement therapies such as dialysis. However, since the rejection of transplanted tissue by the recipient's immune system is one of the most important problems after transplantation, many factors regarding the recipient and donor should be considered before transplantation in order to increase the success of kidney transplant in these patients. Principally, the major histocompatibility complex (MHC) molecules are held responsible for such graft rejection. MHC molecules that present antigen in humans are also called human leukocyte antigen molecules. The antibodies forming against human leukocyte antigens are called anti-human leukocyte antigen (anti-HLA) antibodies. These antibodies may develop in the blood of the recipient after transplantation or they may be already present before transplantation. To reduce the risk of graft rejection, renal allograft recipients are tested to determine whether they have antibodies to donor-specific HLA molecules before transplantation. A lymphocyte cross-matching test is performed for this purpose. In addition, the panel reactive antibody (PRA) test is used to measure the renal transplant candidate's immune sensitivity to HLA molecules other than their own HLA molecules by assessing the diversity of anti-HLA antibodies in the blood of these patients. The PRA test may measure the level of antibodies that the patient's body has produced in response to HLA antigens. PRA testing might be used to estimate the percentage of potential donors in a population who could have the HLA antigens as a potential target of the patient's preexisting anti-HLA antibodies.

The main causes of PRA test positivity include multiparous pregnancy, kidney rejection history, and previous blood transfusions. The reason for anti-HLA antibody detection in the mother in pregnancies might be the formation of antibodies against paternally-inherited fetal antigens. Anti-HLA antibodies can also be seen in men who did not receive blood transfusion or organ transplantation. These anti-HLA antibodies are thought to occur against cross-reactive epitopes found on microorganisms and allergens.

Karahan *et al*., Ayna *et al*. and Ozdemir *et al*. detected PRA positivity in Turkish patients with end-stage renal disease[2-4]. In this study, the aim was to determine PRA values and percentage of PRA positivity in Turkish male patients with CKD, who had not been sensitized by the major known causes (those with no history of organ or tissue transplantation, those with no history of blood transfusion), had not been diagnosed with any autoimmune disease and had not been under immunosuppressive treatment.

Patients and Methods

Patients

A total of 60 male patients aged over 18 years were included in the study. All of the patients were followed up with a diagnosis of CKD at the Nephrology Clinic, Internal Medicine Department, Akdeniz University Medical Faculty Hospital. None of the patients included in the study had been sensitized previously by a known mechanism (they had not received blood transfusion or organ transplantation). All study patients had a glomerular filtration rate (GFR) below 60 mL/min/1.73 m^2. All of the patients were either receiving dialysis treatment or followed up without dialysis treatment. Patient data including age, etiology of CKD, accompanying diseases and dialysis modalities were recorded.

Exclusion criteria

Patients under 18 years of age, female patients, patients with a history of blood transfusion, patients with a history of allograft transplantation, patients with autoimmune diseases, or patients who had received immunosuppressive treatment within the last 6 months were excluded from the study.

Methods

The percentage of HLA antibodies was determined by flow cytometry technique. Two-cc serum samples of all patients were analyzed by flow cytometry. Flow cytometric analyses were performed as outlined below by using the FlowPRA HLA Class I & II Screening Test kits at the Immunology Laboratory, Organ Transplant Institute, Akdeniz University. FlowPRA beads were vortexed prior to use. In second step, 20 µL of test sera were incubated with FlowPRA class I and/or class II beads in 1.5 mL Eppendorf tubes in the dark at 20-25 °C for 30 min with gentle shaking. The 1X solution was then formed by diluting the 10X wash buffer in distilled water. One mL of 1X wash

buffer was added to each tube. The tubes were vortexed again and then centrifuged at 9000 times gravity for 2 minutes. The supernatant in the tubes was aspirated and then discarded. Then, 100 μL of FITC-conjugated goat anti-human IgG *per* test was added to the beads and mixed, after being diluted to 1x with wash buffer from 100X solution. It was incubated for 30 minutes in the dark at 20-25 °C with gentle shaking. Each tube was made ready for analysis by flow cytometry by adding 0.5 mL of wash solution and the results were evaluated with a FACS Conto II device.

Statistical evaluation

Statistical data analysis was performed by using Statistical Package for Social Sciences, Version 22.0 (SPSS 22.0). Student's t-test was used to evaluate the relation between the two categories of PRA (positive and negative) and continuous variables. Fisher exact test and Pearson's χ^2-test were used to evaluate the relation between categorical variables and PRA. The values of p less than 0.05 were considered statistically significant.

The study protocol was approved by the Ethics Committee of the Akdeniz University Faculty of Medicine.

Results

A total of 60 patients were included in the study. Nine out of 60 patients were receiving hemodialysis (HD) treatment and 11 out of 60 patients were receiving peritoneal dialysis (PD) (Table 1). The other 40 patients with CKD were treated with medical treatment, without dialysis treatment.

The etiology of CKD was unknown in 13.3% of all patients (Table 1). Of patients with known etiology, 71.1% had a history of hypertension (HT), 36.5% had a history of type 2 diabetes mellitus (DM), and 9.6% had a history of nephrolithiasis (Table 1). On the other

Table 1. General characteristics of study patients

	Patients
Patient number	60
Mean age (years)	51
Gender (male/female)	60/0
HD/PD	9/11
Etiology: Unknown HT DM Nephrolithiasis Type 2 DM + HT	 13.3% 71.1% 36.5% 9.6% 23%
Accompanying diseases: None HT DM CAD DM + HT Nephrolithiasis Hyperlipidemia RCC	 13.3% 61.7% 31.7% 8.3% 20% 8.3% 8.3% 1.6%
GFR <15 mL/min/1.73 m^2 15<GFR<30 mL/min/1.73 m^2 GFR >30 mL/min/1.73 m^2	58.3% 10% 31.7%

HT = hypertension; PD = peritoneal dialysis; DM = diabetes mellitus; CAD = coronary artery disease; RCC = renal cell carcinoma; GFR = glomerular filtration rate

hand, 8.3% of the study patients had hyperlipidemia and 8.3% had coronary artery disease (CAD) (Table 1). One patient had undergone surgery for renal cell carcinoma (RCC) during childhood. The mean age of study patients was 51 years (Table 1).

The patients included in the study were divided into two groups as PRA negative and PRA positive. PRA positivity was detected in 25% of the patients (Table 2). There were no significant differences between the groups according to mean age, presence of accompanying chronic diseases (hypertension, type 2 diabetes mellitus, hyperlipidemia, nephrolithiasis, coronary artery disease), percentage of hemodialysis patients and percentage of peritoneal dialysis patients (Table 2). On the other hand, evaluation of the GFR levels showed that the PRA positive group contained a significantly higher percentage of end-stage renal disease patients (GFR <15 mL/min/1.73 m^2) as compared with the PRA negative group (p=0.049) (Table 2).

Seventeen (28.3%) study patients were found to be positive for anti-HLA class I antibodies and 16 (26.7%) were found to be positive for anti-HLA class II antibodies on separate analysis for anti-HLA class I and anti-HLA class II antibody positivity (Table 3). Comparison between these patient groups revealed that factors such as low GFR levels, receiving hemodialysis treatment, receiving peritoneal dialysis and presence of accompanying chronic diseases (hypertension, nephrolithiasis, coronary artery disease, type 2 diabetes mellitus) had no effect on anti-HLA class I or anti-HLA class II positivity (Table 3).

In 88.23% of the patients who had anti-HLA class I antibodies, PRA values were within the range of 15%-80%. On the other hand, PRA values were over 80% in 11.77% of the patients positive for anti-HLA class I antibodies. In addition to this, PRA values were within the range of 15%-80% in all patients positive for anti-HLA class II antibodies. PRA values were also within the range of 15%-80% in all patients positive for both anti-HLA class I and class II antibodies. The intergroup comparison to investigate any possible factors related with the percentage of

Table 2. Comparison between PRA negative and PRA positive groups

	Panel reactive antibody (class I/II) Negative	Panel reactive antibody (class I/II) positive	p
Patient number	45 (75%)	15 (25%)	
Mean age (years)	53.15	44.86	0.055
HD (%)	13.3	20	0.678
PD (%)	15.6	26.4	0.442
Etiology:			
Unknown (%)	8.9	26.7	0.098
Accompanying diseases:			
HT (%)	64.4	53.3	0.443
DM (%)	31.1	33.3	0.999
CAD (%)	8.9	6.7	0.999
Type 2 DM + HT (%)	17.8	26.7	0.472
Nephrolithiasis (%)	71.1	8.9	0.591
Hyperlipidemia (%)	11.1	0	0.318
GFR <15 mL/min/1.73 m^2 (%)	51.1	80	0.049
15<GFR<30 mL/min/1.73 m^2 (%)	11.1	6.7	0.999
GFR >30 mL/min/1.73 m^2 (%)	37.8	13.3	0.112

HT = hypertension; PD = peritoneal dialysis; DM = diabetes mellitus; CAD = coronary artery disease; GFR = glomerular filtration rate

PRA level showed that none of the factors including hemodialysis, peritoneal dialysis, presence of accompanying chronic diseases (hypertension, nephrolithiasis, coronary artery disease, type 2 diabetes mellitus) and low GFR levels were related with the percentage of PRA level (Table 4). As the number of patients with

Table 3. Comparison between anti-HLA negative and anti-HLA positive patients

	Anti-HLA					
	Class I			Class II		
	Negative	Positive	p	Negative	Positive	p*
Patient number	43	17		44	16	
HD (%)	11.6	23.5	0.256	13.6	18.8	0.689
PD (%)	16.3	23.5	0.712	15.9	25	0.462
GFR <15 mL/min/1.73 m^2 (%)	51.2	76.5	0.073	52.3	75	0.114
15<GFR<30 mL/min/1.73 m^2 (%)	11.6	5.9	0.665	11.4	6.3	0.999
GFR >30 mL/min/1.73 m^2 (%)	37.2	17.6	0.142	36.4	18.8	0.195
HT (%)	65.1	52.9	0.382	63.6	56.3	0.603
Type 2 DM (%)	30.2	35.3	0.704	31.8	31.3	0.967
Nephrolithiasis (%)	7	11.8	0.616	6.8	12.5	0.602
CAD (%)	7	11.8	0.616	6.8	12.5	0.602
Etiology unknown (%)	9.3	23.5	0.206	9.1	25	0.192

*p values less than 0.05 were considered statistically significant; HT = hypertension; PD = peritoneal dialysis; DM = diabetes mellitus; CAD = coronary artery disease; GFR = glomerular filtration rate

Table 4. Percentage of PRA positivity in patient groups

	Anti-HLA class I 15%-80%	p	Anti-HLA class II 15%-80%	p	Anti-HLA class I-II 15%-80%	p
Patient number	15		16		14	
HD	13.3%	0.999	18.8%	0.689	14.3%	0.999
PD	26.7%	0.442	25%	0.462	28.6%	0.264
GFR <15	73.3%	0.174	75%	0.114	78.6%	0.079
15<GFR<30	6.7%	0.999	6.3%	p=1	7.1%	0.999
GFR >30	20%	0.346	18.8%	0.195	14.3%	0.189
HT	53.3%	0.443	56.3%	0.603	57.1%	0.691
Type 2 DM	40%	0.525	31.3%	0.967	35.7%	0.749
Nephrolithiasis	13.3%	0.591	12.5%	0.602	14.3%	0.582
CAD	6.7%	0.999	12.5%	0.602	7.1%	0.999
Etiology unknown	20%	0.400	25%	0.192	21.4%	0.374

HT = hypertension; PD = peritoneal dialysis; DM = diabetes mellitus; CAD = coronary artery disease; GFR = glomerular filtration rate

PRA positivity over 80% was very low, no analysis was performed in this group. Furthermore, the intergroup comparison to investigate any possible factors related with the percentage of PRA level showed that none of the factors including hemodialysis, peritoneal dialysis, presence of accompanying chronic diseases (hypertension, nephrolithiasis, coronary artery disease, type 2 diabetes mellitus) and GFR levels were related with the percentage of PRA level.

Discussion and Conclusion

Renal transplantation is an important treatment option in patients with end-stage renal disease. Detection of anti-HLA antibodies before transplantation appears to be of vital importance in assessing the possible risk of immune rejection after kidney transplantation. The levels of anti-HLA antibodies in patient sera can be measured by the PRA test. Donor specific anti-HLA antibodies found in the recipient's circulation prior to transplantation may cause hyperacute rejection. In addition, Halloran *et al*. showed that anti-HLA class I antibodies in circulation were related with severe acute rejection[5].

Anti-HLA antibodies can develop in pregnant women, in blood-transfused patients and in patients who received an allograft transplant, and these are the main known sensitization pathways of the immune system against HLA antigens other than the body's own HLA molecules. On the other hand, it is known that the immune system can also develop anti-HLA antibodies without a known history of exposure to foreign HLA antigens[6-8]. Antigenic molecules which had epitopes that can exhibit cross-reactivity with specific HLA molecules may be a reason for developing anti-HLA antibodies spontaneously without exposure to alloantigenic HLA molecules. Such antigenic molecules can enter the body through infections, foods or allergens. Possible molecular similarities between epitopes of foreign antigens and specific HLA molecules may cause the same immune reaction to the foreign antigen to be given to the HLA molecule due to their molecular similarity. In addition, a well-known example of cross-reactivity due to molecular similarity is that antibodies against M proteins of the streptococcal bacterium cross-react with cardiac myosin proteins[9,10]. Furthermore, Katerinis *et al*. report that multiple dose influenza vaccinations in kidney transplant recipients may be associated with anti-HLA antibody development in a significant portion of patient population[11]. In our study, patients with transfusion history, allograft recipients, and women patients (in order to completely exclude the pregnancy history) were excluded. Because the immune reaction may go beyond normal functions in autoimmune diseases and because the autoimmune reactions may develop against other self antigens of the body, including HLA molecules, we also excluded patients with autoimmune diseases. In fact, Yamagiwa *et al*. report that anti-HLA class II antibodies were frequently found in autoimmune hepatitis cases[12].

In our study, PRA positivity was investigated by flow cytometry technique in male patients who had no known risk factors for anti-HLA antibody development, and PRA positivity was detected in 25% of the patients. In addition, 28.3% of patients had anti-HLA class I antibodies and 26.7% of them had anti-HLA class II antibodies. When we look at the literature, we see that Morales-Buenrostro *et al*. detected anti-HLA antibodies in 63% of 424 healthy males[6]. Karahan *et al*. found PRA positivity in 110 of 674 patients who were followed-up with end-stage renal disease[2]. However, the study by Karahan *et al*. included patients who had a possible alloantigenic HLA exposure history[2]. Özdemir *et al*. found PRA positivity in 33.7% of patients who were receiving hemodialysis therapy due to end-stage renal failure[13]. However, PRA levels over 30% were accepted as PRA positive in the study by Özdemir *et al*.[13]. In addition to this, the study by Özdemir *et al*. included female patients, patients with a history of allograft kidney rejection, and patients with blood transfusion history[13]. Unlike our study, the previous works by Karahan *et al*. and Özdemir *et al*. did not exclude factors that may contribute to the formation of anti-HLA antibodies, such as pregnancy, blood transfusion, and allograft transplantation history[2,13].

In our study, we also compared PRA positivity, which is accepted as a sign of the presence of HLA sensitization, and PRA negativity, which is accepted as a sign of absence of HLA sensitization, in terms of dialysis modality, GFR levels, mean age, and presence of accompanying chronic diseases (HT, DM, nephrolithiasis). We found that the PRA positive group had a significantly higher percentage of end-stage renal disease patients as compared to the PRA negative group. However, no similar correlation was observed in the prevalence of patients receiving peritoneal dialysis or hemodialysis treatment. Besides, Hung *et al*. demonstrated that the prevalence of PRA positivity was higher in patients with end-stage renal disease

as compared to healthy controls[14]. In addition to this, Pour-Reza-Gholi *et al*. showed a correlation between age and PRA level[15]. On the other hand, we found no difference between the PRA positive group and PRA negative group in terms of mean age.

It is known that the presence of a high level of anti-HLA antibodies in patients waiting for kidney transplantation may increase the patient waiting time, as well as the possibility of rejection in post-transplant period, and impair graft survival[16,17]. In our study, PRA levels over 80% were classified as high sensitization and PRA values were over 80% in 11.77% of the patients positive for anti-HLA class I antibodies. Due to the higher risk of post-transplant rejection in highly sensitized patients, this group of patients should be treated with desensitization therapies in order to eliminate or reduce the potential donor-specific HLA alloantibodies prior to transplantation.

In conclusion, PRA levels of the candidates for kidney transplantation should always be measured to assess their state of sensitization before transplantation, even though they have no risk factors known to cause anti-HLA antibody development.

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Sažetak

ODREĐIVANJE POSTOTKA POZITIVNOSTI NA PRA KOD MUŠKIH BOLESNIKA S KRONIČNOM BUBREŽNOM BOLEŠĆU TEHNIKOM PROTOČNE CITOMETRIJE

F. Avcı Merdin, H. Koçak i *S. Köksoy*

Protutijela usmjerena protiv molekula humanog leukocitnog antigena (*human leukocyte antigen*, HLA), koji ima bitnu ulogu u histokompatibilnosti alografta, nazivaju se anti-HLA protutijela. Anti-HLA protutijela protiv molekula stranog antigena mogu biti prisutne u bolesnika s kroničnom bubrežnom bolešću (KBB) čak i prije transplantacije. Test panela reaktivnih protutijela (*panel reactive antibody*, PRA) rabi se za mjerenje imunosne osjetljivosti kandidata za transplantaciju bubrega na druge molekule HLA osim onih vlastitih kroz procjenu raznolikosti anti-HLA protutijela u krvi ovih bolesnika. Cilj ovog istraživanja bio je utvrditi vrijednosti PRA i postotak pozitivnosti na PRA kod muških turskih bolesnika s KBB koji nisu bili senzibilizirani glavnim poznatim uzrocima (oni bez anamneze transplantacije organa ili tkiva, oni bez anamneze transfuzije krvi), koji nisu imali dijagnozu bilo kakve autoimune bolesti i koji nisu uzimali imunosupresivnu terapiju. U istraživanje je bilo uključeno 60 muških bolesnika u dobi iznad 18 godina. Svi bolesnici praćeni su s dijagnozom KBB u Nefrološkoj ambulanti Klinike za unutarnje bolesti, Sveučilišna bolnica Akdeniz. Svi bolesnici uključeni u istraživanje nisu bili prethodno senzibilizirani poznatim mehanizmima, odnosno nisu primili transfuziju krvi ili transplantaciju organa. Stopa glomerularne filtracije (*glomerular filtration rate*, GFR) kod svih je bolesnika bila ispod razine od 60 mL/min/1,73 m^2. Zabilježeni su sljedeći podatci svakog bolesnika: dob, etiologija KBB, prateće bolesti, podatci o vrsti dijalize. Postotak HLA protutijela utvrđen je tehnikom protočne citometrije. Statistička analiza podataka provedena je pomoću SPSS 22.0, a vrijednosti p manje od 0,05 smatrane su statistički značajnima. Od svih bolesnika 20 ih je primalo liječenje dijalizom zbog zadnjeg stadija bubrežne bolesti. Od 60 bolesnika uključenih u istraživanje 25% ih je imalo nalaz pozitivan na PRA. Zasebna analiza na anti-HLA protutijela I. i II. klase pokazala je da je 28,3% bolesnika pozitivno na anti-HLA protutijela I. klase, a 26,7% bolesnika na anti-HLA protutijela II. klase. Kad su bolesnici podijeljeni u dvije skupine pozitivnih i negativnih na PRA nije bilo razlika između dviju skupina u dobi, postotku bolesnika na hemodijalizi, postotku bolesnika na peritonejskoj dijalizi i prisutnosti pratećih kroničnih bolesti (npr. hipertenzija, dijabetes melitus tip 2, hiperlipidemija, nefrolitijaza, bolest koronarnih arterija). Uz to, procjena razina GFR pokazala je da je u skupini bolesnika pozitivnih na PRA bio značajno viši postotak bolesnika sa zadnjim stadijem bubrežne bolesti (GFR <15 mL/min/1,73 m^2) u usporedbi sa skupinom bolesnika negativnih na PRA. Podrobna analiza postotaka razina PRA kod bolesnika pozitivnih na PRA, koja je provedena kako bi se utvrdio stupanj senzibilizacije, pokazala je vrijednosti PRA iznad 80% u 11,77% bolesnika pozitivnih na anti-HLA protutijela I. klase. S druge strane, kod 88,23% bolesnika s HLA protutijelima II. klase vrijednosti PRA bile su u rasponu od 15% do 80%. Vrijednosti PRA ispod 80% utvrđene su kod svih bolesnika pozitivnih na anti-HLA protutijela II. klase i onih pozitivnih na anti-HLA protutijela I. i II. klase. Zaključno, razine PRA kod kandidata za transplantaciju bubrega treba uvijek izmjeriti kako bi se procijenio njihov status senzibilizacije prije transplantacije, čak i onda kad nemaju nikakvih rizičnih čimbenika koji bi uzrokovali razvoj anti-HLA protutijela.

Ključne riječi: *Kronična bubrežna bolest; Protočna citometrija; Anti-HLA protutijela; Transplantacija bubrega*