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PATRICIA KEIKO SAITO

Polimorfismo HLA e resposta imune humoral aos antígenos HLA em candidatos ao transplante renal da região Norte/Noroeste do Estado do Paraná

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2017

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutor em Ciências da Saúde.
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EPÍGRAFE

"O êxito ou o fracasso de uma pessoa depende da habilidade com que ela utiliza o tempo".

(MASSAHARU TANIGUCHI)

Polimorfismo HLA e resposta imune humoral aos antígenos HLA em candidatos ao transplante renal da região Norte/Noroeste do Estado do Paraná

RESUMO

O sistema HLA (*Human Leukocyte Antigen*) possui papel de destaque entre os sistemas biológicos envolvidos no processo de rejeição. Nesse trabalho, foram avaliadas as frequências alélicas HLA de classe I (HLA-A, -B e -C) e classe II (HLA-DRB1, -DQA1 e -DQB1) e a resposta imune humoral aos antígenos HLA em pacientes renais crônicos, candidatos ao transplante renal, da região Norte/Noroeste do Estado do Paraná. A tipificação HLA foi realizada pelo método de reação em cadeia da polimerase-sequência específica de oligonucleotídeos (PCR-SSO), associado à tecnologia Luminex, utilizando DNA genômico extraído de leucócitos do sangue periférico. A avaliação da presença de anticorpos anti-HLA contra um painel de antígenos HLA (*Panel-Reactive Antibodies* – PRA) foi realizada pelo método de citotoxicidade dependente de complemento (CDC), CDC com a adição de antiglobulina humana (CDC-AGH), CDC com a adição de ditiotreitol (CDC-DTT) e imunoensaio de fase sólida associado à tecnologia Luminex (IFS; kit LS1PRA, One Lambda, Inc.), utilizando amostras de soro. A determinação das especificidades de anticorpos anti-HLA foi realizada pelos kits LS1PRA e LS2PRA (One Lambda, Inc.). O desempenho dos métodos de CDC, CDC-AGH, CDC-DTT e IFS para detecção de anticorpos anti-HLA classe I foi analisado em 70 amostras de soro de pacientes renais crônicos. A média de PRA detectada pelo IFS (37,5 ± 34,2%) foi maior do que os valores detectados pelos outros métodos. Análises comparativas revelaram diferença significativa entre CDC e CDC-AGH (P <0,001), e entre CDC e IFS (P <0,001), mas não entre CDC-AGH e IFS (P = 0,803). O desempenho do método CDC-AGH para a detecção de anticorpos anti-HLA foi comparável ao IFS na avaliação de PRA classe I. A influência dos grupos alélicos HLA de classe I (HLA-A, -B, -C) e classe II (HLA-DRB1, -DQA1, -DQB1) na resposta imune humoral aos antígenos HLA foi estudada em 319 pacientes renais crônicos (198 homens e 121 mulheres). Do total de pacientes, 63,6% apresentaram PRA positivo. Positividade de PRA foi significativamente associada ao sexo feminino (P <0,001), transfusões (P <0,001) e gravidez (P <0,001). As frequências de *HLA-B*14* (OR: 3,32; IC: 1,13-9,76), *HLA-C*08* (OR: 3,98; IC: 1,38-12,38) e *HLA-DRB1*16* (OR: 3,32; IC: 1,13-9,76) foram significativamente maiores em pacientes com PRA negativo em comparação com pacientes com PRA positivo, sugerindo que grupos alélicos HLA de classe I (*HLA-B*14, -C*08*) e classe II (*HLA-DRB1*16*) podem

estar envolvidos na diminuição da resposta imune humoral aos antígenos HLA. A sensibilização aos antígenos HLA e o histórico de transfusão sanguínea foram avaliados em 236 pacientes renais crônicos do sexo masculino que aguardam seu primeiro transplante renal. Do total de pacientes, 121 (51,3%) apresentaram PRA positivo e 138 (58,5%) apresentaram história prévia de transfusão sanguínea. Histórico de transfusão foi significativamente diferente entre os grupos PRA positivo e PRA negativo (P <0,001). A sensibilização aos antígenos HLA por transfusão ocorreu em 88 (37,3%) pacientes. Pacientes transfundidos apresentaram maior tempo de espera em diálise quando comparados aos pacientes não transfundidos (P <0,01). Positividade de PRA classe I ou/e II e média de anticorpos anti-HLA específicos classe I ou/e II foram maiores para aqueles que receberam transfusão em comparação aos que não receberam transfusão (P <0,05). Muitos pacientes continuam a receber transfusões sanguíneas antes do transplante, aumentando a possibilidade de se tornarem sensibilizados. Este estudo propiciou o conhecimento do polimorfismo HLA e da resposta imune humoral aos antígenos HLA em candidatos ao transplante renal da região Norte/Noroeste do Estado do Paraná.

Palavras-chave: Antígenos HLA. Anticorpos. Imunoensaio. Teste imunológico de citotoxicidade. Teste de histocompatibilidade. Imunidade humoral. Transfusão de sangue. Insuficiência Renal Crônica. Transplante renal.

HLA polymorphism and humoral immune response to HLA antigens in renal transplant candidates from North/Northwestern of Parana State

ABSTRACT

The HLA systems (Human Leukocyte Antigen) have a prominent role among the biological systems involved in the rejection process. In this study, we evaluated the HLA class I (HLA-A,-B and-C) and class II (HLA-DRB1, -DQA1 and -DQB1) allele frequencies and humoral immune response to HLA antigens in chronic renal patients, renal transplant candidates, from Northern/Northwestern of Parana State. HLA typing was performed by the method of polymerase chain reaction-sequence specific primers (PCR-SSO) associated with Luminex technology, using genomic DNA extracted from peripheral blood leukocytes. The evaluation of the presence of anti-HLA antibodies against a panel of HLA antigens (Panel-Reactive Antibodies - PRA) was performed by complement dependent cytotoxicity (CDC), CDC with the addition of anti-human globulin (CDC-AHG), CDC with the addition of dithiothreitol (CDC-DTT) and solid-phase immunoassay associated with Luminex technology (SPI; LS1PRA kit, One Lambda, Inc.), using serum samples. Determination the specificities of anti-HLA antibodies were performed by kits LS1PRA and LS2PRA (SPI; One Lambda, Inc.). The performance of CDC, CDC-AHG, CDC-DTT and SPI methods for detection of anti-HLA antibodies class I was analyzed in 70 serum samples from chronic renal patients. Mean PRA detected by SPI (37.5 ± 34.2%) was higher than the values detected by the other methods. Comparative analyses revealed significant difference between CDC and CDC-AHG (P <0.001), and between CDC and SPI (P <0.001), but not between CDC-AHG and SPI (P = 0.803). The performance of the CDC-AHG method for detection of anti-HLA antibodies was comparable to the SPI in the evaluation of percent class I PRA. The influence of HLA class I (HLA-A, -B, -C) and class II (HLA-DRB1, -DQA1, -DQB1) allele groups on humoral immune response to HLA antigens were studied in 319 chronic renal patients (198 males and 121 females). Of the total patients, 63.6% had positive PRA. PRA-positivity was significantly associated with female gender (P <0.001), transfusions (P <0.001) and pregnancies (P <0.001). The frequencies of *HLA-B*14* (OR: 3.32; CI: 1.13-9.76), *HLA-C*08* (OR: 3.98; CI: 1.38-12.38) and *HLA-DRB1*16* (OR: 3.32; CI: 1.13-9.76) were significantly higher in patients with negative PRA compared with patients with positive PRA, suggesting that HLA class I (*HLA-B*14, -C*08*) and class II (*HLA-DRB1*16*) allele groups might be involved in the decrease of humoral immune response to HLA antigens. The sensitization to HLA

antigens and history of blood transfusion was evaluated in 236 chronic renal male patients awaiting their first kidney transplant. Of the total patients, 121 (51.3%) had positive PRA and 138 (58.5%) had previous history of blood transfusion. Transfusion history showed a significant difference between the PRA-positive and PRA-negative group (P <0.001). Sensitization to HLA antigens from transfusion occurred in 88 (37.3%) patients. Transfused patients had longer waiting times on dialysis when compared with nontransfused patients (P <0.01). Positivity of PRA class I or/and class II and median number of HLA class I or/and class II specific antibody HLA-specific were higher for those who received transfusion compared to those who did not received transfusion (P <0.05). Many patients continue to receive blood transfusions before transplantation, increasing the possibility of becoming sensitized. This study allowed the knowledge of HLA polymorphism and humoral immune response to HLA antigens in renal transplant candidates from North/Northwestern of Parana State.
Keywords: HLA Antigens. Antibodies. Immunoassay. Immunologic Cytotoxicity Tests. Histocompatibility Testing. Humoral Immunity. Blood Transfusion. Chronic Kidney Failure. Kidney Transplantation.

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1. CAPÍTULO I

1.1. COMPLEXO PRINCIPAL DE HISTOCOMPATIBILIDADE

O Complexo Principal de Histocompatibilidade (CPH) ou MHC (do inglês, *Major Histocompatibility Complex*) é uma extensa região formada por genes polimórficos, responsáveis por codificar moléculas que participam de importantes processos da resposta imune. O MHC foi descoberto como um loco gênico, por meio de pesquisas conduzidas na década de 1930, por Peter Gorer (GORER, 1936; GORER, 1937) e na década de 1940, por George Snell (SNELL, 1948). Estes pesquisadores investigavam a rejeição de tumores e de tecidos normais alogênicos, em linhagens de camundongos. O loco, em especial, responsável pela rejeição de tecidos enxertados estava localizado no cromossomo 17 de camundongos e codificava um grupo sanguíneo polimórfico, denominado antígeno II. Consequentemente, essa região foi chamada de histocompatibilidade-2 (H-2) (ABBAS et al., 2015).

Os genes do MHC estão presentes em todos os mamíferos, e são homólogos aos genes do H-2 identificados em camundongos. Em 1954, Dausset, observando indivíduos politransfundidos, verificou que, em seus soros estavam presentes anticorpos capazes de aglutinar, quando entravam em contato com leucócitos de outros indivíduos (DAUSSET, 1954). Baseado nesta descoberta, em 1958, Dausset, por meio da técnica de leucoaglutinação no soro de politransfundidos, descreveu o primeiro antígeno pertencente ao que, mais tarde, seria chamado de sistema HLA. Esse antígeno recebeu o nome de "Mac", correspondente ao atual antígeno HLA-A2 (DAUSSET, 1958; TERASAKI; PARK, 2000).

Ainda em 1958, outros pesquisadores obtiveram descobertas semelhantes, por meio da observação de anticorpos contra leucócitos, no soro de mulheres multíparas (VAN ROOD et al., 1958; PAYNE; ROLFS, 1958).

A partir da década de 1960, diversos pesquisadores tiveram interesse pela investigação do MHC. Para que fossem discutidos os resultados obtidos por diferentes centros de pesquisa, e a padronização, tanto dos testes utilizados quanto a nomenclatura do sistema HLA, foram criados os Encontros Internacionais de Histocompatibilidade ou IHWS (do inglês, *International Histocompatibility Workshop*). Foi assim que, a partir de 1964, iniciou-se uma série desses encontros, com a finalidade de atualizar continuamente as descobertas, nas áreas de imunogenética e histocompatibilidade. Ao longo dos IHWS foi definido que, uma região do MHC, em humanos, receberia o nome de HLA (do inglês, *Human Leucocyte Antigen*), e a primeira especificidade HLA, identificada por Dausset, e depois por outros pesquisadores,

receberia o nome de HL-A2. Em 1967, foi estabelecido o Comitê de Nomenclatura dos fatores do sistema HLA da Organização Mundial da Saúde (OMS), responsável pela normatização da nomenclatura do sistema HLA (NOMENCLATURE, 1968). A comunidade científica, ao longo dos vários IHWS, caracterizou, estruturalmente e funcionalmente, vários genes pertencentes ao sistema HLA.

O MHC, localizado no braço curto do cromossomo 6 humano (segmento 6p21.3), é didaticamente divido em 3 regiões: região de classe I, II e III (Figura 1). Cada região é constituída de diversos locos, os quais contêm genes que codificam os antígenos ou moléculas HLA de classe I e II. Tais moléculas são glicoproteínas altamente polimórficas, que diferem entre si quanto à distribuição em tecidos, estrutura e funções (ABBAS et al., 2015).

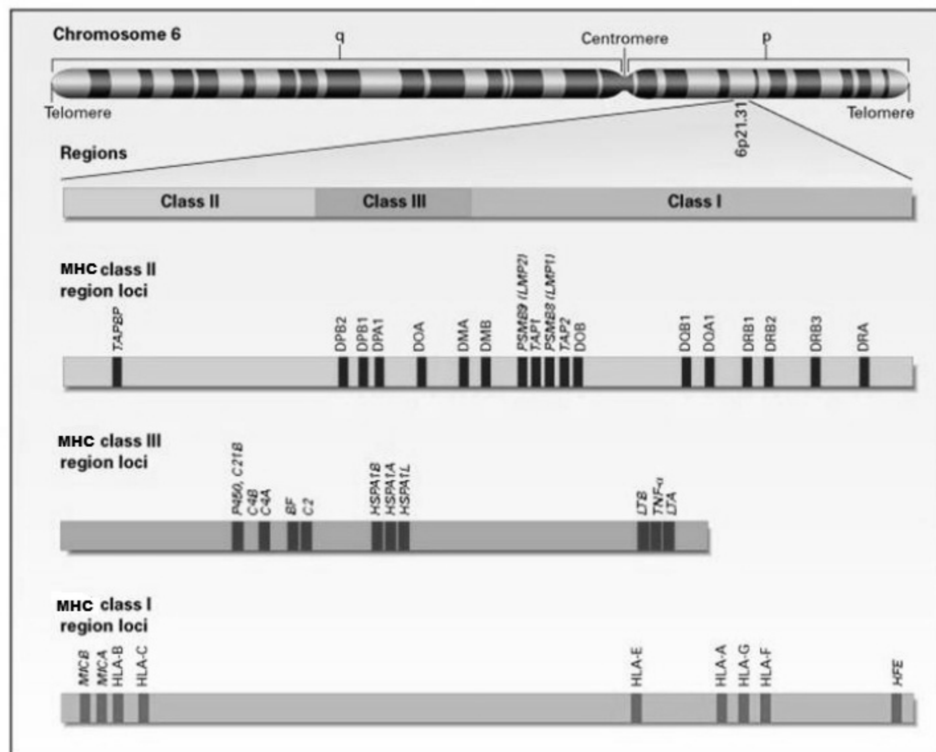

Figura 1. Estrutura gênica do MHC (*Major Histocompatibility Complex*) (adaptado de KLEIN; SATO, 2000).

Os genes da região de classe I codificam as moléculas clássicas HLA-A, -B e -C, presentes em células nucleadas e plaquetas, portanto, em quase todas as células somáticas do organismo. Elas se ligam aos receptores de linfócitos TCD8+, e possuem papel relevante na resposta imune, detectando alterações de expressão nas células, devido a infecções ou ao desenvolvimento de células tumorais. Durante a transformação celular, alterações na expressão do MHC de Classe I provocam a ativação das células *Natural Killer* (NK) que

levam a lise das células infectadas ou tumorais. Os genes da região de classe I codificam também as moléculas não clássicas HLA-E, -F e –G que parecem participar no reconhecimento dos antígenos pelas células NK; e HLA-H que pode estar envolvido no metabolismo do ferro, sem função conhecida no sistema imunológico (LEWIN, 2009).

As moléculas HLA-E são expressas em quase todos os tecidos e estão envolvidas principalmente na regulação da imunidade inata. Estudos recentes apontam a colaboração de HLA-E na ligação com células T e na resposta imune após transplantes (SULLIVAN et al., 2008). As moléculas HLA-F e -G possuem funções ainda pouco definidas, polimorfismos limitados e expressões restritas a determinados tipos celulares. O HLA-F é expresso em tecidos fetais, e o HLA-G predomina na interface materno-fetal. As moléculas HLA-G estão envolvidas na implantação do blastocisto, angiogênese placentária e principalmente na modulação do sistema imune materno durante a gravidez e consequentemente na aceitação do feto semi-alogênico (JURISICOVA et al., 1996).

Estruturalmente, as moléculas de classe I são constituídas por uma cadeia polipeptídica pesada (cadeia α), codificada na região do MHC, de aproximadamente 44 a 47 kD, a qual se encontra ligada não covalentemente a uma proteína não polimórfica, codificada por um gene localizado no cromossomo 15 humano (β2-microglobulina de 12 kD) (ENGELHARD, 1994).

Os genes da região de classe II codificam as moléculas HLA-DR, -DQ e -DP. As moléculas de classe II possuem uma distribuição mais restrita, se comparadas as de classe I, sendo encontradas, principalmente na superfície de células que participam diretamente da resposta imune, tais como macrófagos, monócitos, células dendríticas, células de Langerhans, linfócitos B e linfócitos T ativados. Elas se ligam aos receptores de linfócitos TCD4+ e permitem, assim, a ativação dessas células por apresentarem peptídeos derivados de organismos e proteínas extracelulares. Portanto, as moléculas do MHC desempenham papel importantíssimo na apresentação de antígenos. A variabilidade HLA pode apresentar o peptídeo de forma diferente e induzir respostas diversas. Estruturalmente as moléculas de classe II são constituídas de duas cadeias polipeptídicas ligadas não covalentemente, uma cadeia α com 32 a 34 kD e uma cadeia β de 29 a 32 kD. Ao contrário das moléculas de classe I, ambas as cadeias das moléculas de classe II são codificadas por genes do MHC (BROWN et al., 1993).

Os genes da região de classe III não codificam moléculas HLA, porém, codificam componentes do sistema complemento (C2, C4, fator B), enzima 21-hidroxilase, fator de necrose tumoral (TNFs), entre outros (ABBAS et al., 2015).

Os alelos da região do MHC são expressos codominantemente. O conjunto de alelos proximais que segregam juntos, presentes em um mesmo cromossomo, é chamado de haplótipo. Todo indivíduo herda dois haplótipos, sendo um de origem materna e outro de origem paterna (ABBAS et al., 2015).

1.2. **IMUNOLOGIA DOS TRANSPLANTES**

1.2.1. **RECONHECIMENTO DE ANTÍGENOS**

O transplante de tecidos e órgãos é uma alternativa terapêutica para uma variedade de doenças. Uma grande limitação, para o sucesso do transplante, é a resposta imune do receptor ao tecido ou órgão do doador. Quando um órgão ou tecido alogênico é transplantado em um receptor, este é reconhecido como estranho pelo seu sistema imune. O reconhecimento de antígenos transplantados, como próprias ou não-próprias, é determinado principalmente pelas moléculas HLA, por serem altamente imunogênicas. Esse processo é conhecido como alorreconhecimento, e tem como evento central o reconhecimento do antígeno do enxerto, via MHC pelo receptor de célula T (TCR) (SHERMAN; CHATTOPADHYAY, 1994).

O alorreconhecimento pode ocorrer por duas vias distintas: a via direta e a via indireta. Ambas as vias de alorreconhecimento podem ocorrer simultaneamente, ou em tempos diferentes, no processo de rejeição. A via direta de alorreconhecimento envolve o reconhecimento do MHC-peptídeo intacto na superfície das células apresentadoras de antígenos (APCs) do enxerto, por linfócitos T do receptor. A via indireta envolve o reconhecimento pelos linfócitos T do receptor, de peptídeos do doador processados e apresentados via MHC pelas APCs do receptor. O processo de alorreconhecimento, por meio das vias direta ou indireta, desencadeia ativação de linfócitos T e pode resultar na rejeição do enxerto (SHERMAN; CHATTOPADHYAY, 1994).

Tanto os linfócitos T CD4+ quanto os linfócitos T CD8+ são capazes de mediar a rejeição, por meio de mecanismos diferentes. Os linfócitos T CD4+ se diferenciam em células efetoras, produtoras de citocinas, que irão lesar o enxerto e ativar os linfócitos B a produzir anticorpos anti-HLA. Os linfócitos T CD8+ se diferenciam em linfócitos citotóxicos, com

potencialidade de destruir as células do enxerto, que expressam moléculas HLA classe I (ABBAS et al., 2015).

1.2.2. **REJEIÇÃO DE ENXERTOS**

A rejeição de enxerto é, em geral, definida como uma deterioração da função do enxerto associada a alterações histopatológicas. De acordo com as respostas efetoras contra o enxerto é possível classificar as rejeições, a qual leva também em consideração o decurso temporal desta resposta e as características histopatológicas observadas. Desta forma, é possível considerar os padrões de rejeição como hiperaguda, aguda e crônica (CAI; TERASAKI, 2005).

A resposta efetora no processo da rejeição pode ser dividida em dois grupos: resposta imune celular e resposta imune humoral. Porém a distinção entre os processos celulares e humorais, nem sempre é evidente, sendo que, na maioria das rejeições, ambos os processos podem atuar concomitantemente. A rejeição mediada por células pode se manifestar como uma rejeição aguda. Em contrapartida, a rejeição mediada por anticorpos pode se manifestar como uma rejeição hiperaguda, aguda ou crônica (CAI; TERASAKI, 2005; ABBAS et al., 2015).

A rejeição hiperaguda é caracterizada por oclusão trombótica dos vasos do enxerto, e tem início dentro de minutos ou horas, depois que os vasos do receptor são anastomosados com os do enxerto. É normalmente causada por anticorpos pré-existentes, na circulação do receptor, os quais se ligam ao endotélio do enxerto (MICHAELS et al., 2003; CAI; TERASAKI, 2005). A rejeição hiperaguda leva a perda de função do enxerto e a única forma de preveni-la é realizando a prova cruzada (*crossmatch*) entre receptor e doador. A rejeição aguda é um processo de dano parenquimal e vascular, mediado por linfócitos T e anticorpos, e ocorre, normalmente, depois da primeira semana de transplante. Na rejeição aguda, mediada por anticorpos, geralmente ocorre disfunção do enxerto rapidamente. O processo de rejeição crônica é caracterizado por fibrose e anormalidades vasculares, com perda da função do enxerto, que ocorre durante um período prolongado. Na rejeição crônica, mediada por anticorpos, a disfunção do enxerto ocorre mais lentamente e, geralmente, é acompanhada de proteinúria (ABBAS et al., 2015).

1.2.3. **SENSIBILIZAÇÃO AOS ANTÍGENOS HLA**

A resposta imune ao enxerto inicia-se com uma fase de sensibilização, para que os linfócitos reativos aos antígenos de histocompatibilidade sejam ativados, proliferam e diferenciam-se em células efetoras, e uma fase efetora, na qual ocorre o ataque do sistema imune contra o enxerto, podendo ocorrer de forma humoral ou celular (ABBAS et al., 2015). A resposta imune humoral aos aloantígenos HLA desempenha um papel importante na rejeição, seja por anticorpos pré-formados (rejeição hiperaguda) ou formados após o transplante (rejeições agudas ou crônicas). Os indivíduos que desenvolvem anticorpos anti-HLA são denominados sensibilizados e representam um número expressivo dentro da lista de espera em um centro de transplante (SAITO et al., 2014).

Desde a década de 1960 já se conhece a importância dos anticorpos anti-HLA na rejeição do enxerto (KISSMEYER-NIELSEN et al., 1966). Vários estudos evidenciaram que quase todos os indivíduos que sofreram uma rejeição hiperaguda ou aguda de um órgão, sendo este o primeiro enxerto ou retransplante, podem apresentar em seus soros anticorpos anti-HLA (PATEL; TERASAKI, 1969; HALLORAN, 1990). Estes anticorpos podem ser formados, em decorrência de uma sensibilização prévia ao transplante. A exposição a aloantígenos HLA pode ocorrer durante a gestação ou abortos (VAN ROOD et al., 1958; PAYNE; ROLFS, 1958; VONGWIWATANA et al., 2003), após uma transfusão sanguínea (DAUSSET, 1954; VONGWIWATANA et al., 2003) ou a um transplante prévio (THICK et al., 1984; VONGWIWATANA et al., 2003). A sensibilização alogênica pelo enxerto é um resultado de uma cooperação celular entre Linfócitos T e B, que ocorre principalmente após reconhecimento indireto dos aloantígenos pelo linfócito T CD4+ e pelo receptor de célula B (BCR) nos linfócitos B, resultando na produção de anticorpos (ABBAS et al., 2015).

1.2.4. **DETECÇÃO DE ANTICORPOS ANTI-HLA**

Os anticorpos anti-HLA desempenham um papel fundamental na sobrevida dos enxertos, por serem altamente reativos. Em virtude da existência de anticorpos específicos contra o doador na fase pré-transplante estar diretamente envolvida com a patogênese da rejeição hiperaguda (KISSMEYER-NIELSEN et al., 1966), a presença desses anticorpos é sempre avaliada antes de um transplante de órgão sólido, pela realização da prova cruzada (*crossmatch*), sendo a única forma preventiva da rejeição hiperaguda. Além deste teste, rotineiramente é realizada a detecção de anticorpos anti-HLA pelo teste de PRA (Reatividade contra Painel), visando monitorar o grau de sensibilização dos pacientes em lista de espera para um transplante.

Desde a década de 1950, a sorologia formou a base dos testes clínicos de investigação HLA. Esses testes melhoraram significativamente, quando o ensaio de leucoaglutinação foi substituído pelo teste de CDC (microlinfocitotoxicidade dependente de complemento), desenvolvido por Terasaki e McClelland em 1964 (TERASAKI; MCCLELLAND, 1964).

Historicamente, o teste padrão ouro para detecção de anticorpos anti-HLA é o teste de CDC. O CDC pode ser utilizado para realização da prova cruzada (*crossmatch*), sendo utilizado, até hoje, na investigação pré-transplante. Por meio desse teste, utilizando o soro do receptor e células mononucleares do doador, é possível detectar se um paciente possui anticorpos anti-HLA fixadores de complemento específicos contra um potencial doador. Anticorpos doador-específico (DSA), detectados por meio da prova cruzada realizada pelo teste CDC, ou seja, prova cruzada positiva, sugere contraindicação ao transplante (GEBEL et al., 2003), sendo importante para evitar a rejeição hiperaguda, e perda imediata do enxerto (PATEL; TERASAKI, 1969).

Outro teste de investigação pré-transplante é o teste de PRA (Reatividade contra Painel), que verifica o grau de sensibilização, geralmente em percentual, em relação a um painel de antígenos HLA. Para realizar este teste, o mesmo teste de CDC da prova cruzada é realizado, porém, utiliza-se um painel de diferentes células (e não somente do doador), que é representativo da distribuição de antígenos HLA, na população. Sendo que, um número percentual define a reatividade existente a esse painel de células. Esse percentual varia de 0% (não sensibilizado ou não produtor de anticorpos) a 100% (hipersensibilizado ou produtor de anticorpos) (TRIVEDI et al., 2007).

O teste de PRA pelo teste de CDC consiste em promover uma reação "*in vitro*", onde ocorre uma mistura do soro do receptor com um painel pré-selecionado de células (linfócitos T) de indivíduos com tipagem HLA diversificada. Esta mistura de células e soro permanece em período de incubação e subsequentemente adiciona-se complemento de coelho. A ligação do anticorpo presente no soro e do antígeno correspondente na superfície dos linfócitos promove a fixação do complemento, culminando na ativação do sistema complemento e consequente morte celular (lise). Tal resultado corresponde a uma reação positiva. A morte celular pode ser visualizada adicionando-se um corante vital ao meio de cultura. As células que sofrerem lise celular irão incorporar a cor do corante. A ausência de anticorpos no soro não permite a fixação do complemento e, desta forma, as células vivas mantêm suas membranas intactas e por isso não incorporam o corante adicionado ao meio, caracterizando desta forma, um resultado negativo. O valor porcentual da PRA é estimado por meio do

número de resultados positivos dividido pelo número de células ao qual o soro do paciente foi submetido, multiplicado por 100 (DYER; MIDDLETON, 1993).

O teste de CDC com adição de anti-imunoglobulina humana (AGH) surgiu para aprimorar a técnica de CDC clássica (NELKEN et al., 1970; JOHNSON et al., 1972; FULLER et al., 1982; ZACHARY et al., 1995). Devido à baixa sensibilidade do teste de CDC, a utilização de AGH possibilitou o aumento da reação de citotoxicidade promovida por anticorpos fixadores de complemento. Neste teste, a reação básica consiste em incubar uma mistura de células (linfócitos) com o soro do receptor e após este período adiciona-se a AGH, em seguida acrescenta-se o complemento. A adição de AGH na reação de CDC confere ao teste maior sensibilidade e é capaz de detectar anticorpos fixadores de complemento com título baixo no soro dos pacientes (NELKEN et al., 1970, GEBEL; LEBECK et al., 1991). A interpretação dos resultados por meio deste teste segue o mesmo padrão da leitura do teste de CDC clássico.

O teste de CDC após tratamento do soro com dithiotreitol (DTT) é outro aprimoramento da técnica de CDC clássica, possibilitando distinguir a qual isotipo o anticorpo presente pertence (IgG ou IgM). A maioria dos anticorpos anti-HLA são da classe IgG, enquanto que os anticorpos da classe IgM são normalmente, mas não exclusivamente autoanticorpos (KHODADADI et al., 2006).

Na biologia dos transplantes existem duas classes de imunoglobulinas que devem ser avaliadas durante o processo de seleção para identificar o par adequado (receptor/doador). A primeira classe de imunoglobulina avaliada é a IgG e a segunda é a IgM. A IgG possui uma estrutura monomérica pesando aproximadamente 150.000 daltons; sua detecção no soro de pacientes candidatos a um transplante aponta que este indivíduo possui um alto grau de sensibilização contra os aloantígenos HLA, evidenciando um possível risco de rejeição hiperaguda (PATEL; TERASAKI, 1969; OZDEMIR et al., 2004). A segunda molécula é a imunoglobulina M (IgM) que possui uma estrutura pentamérica e pesa aproximadamente 900.000 daltons. Estas moléculas podem ser detectadas por meio da reação de citotoxicidade associada ao uso de um agente redutor (DTT - dithiotreitol). O DTT atua quebrando as pontes dissulfeto das moléculas de IgM (TAYLOR et al., 1989; TEN HOOR et al., 1993; ZACHARY et al., 1995; DYER; MIDDLETON, 1993).

Se em um teste de CDC ou CDC+AGH o resultado for positivo, isto se deve a existência de anticorpos IgG ou IgM. Para diferenciar a classe de anticorpos existentes, pode-se utilizar o DTT, desta forma as moléculas de IgM serão quebradas restando apenas

moléculas da classe IgG, assim se o teste após tratamento do soro com DTT permanecer positivo, isto indica que a positividade era decorrente de anticorpos da classe IgG, mas se o teste após o tratamento se tornar negativo, isto significa que a positividade era devido a presença de anticorpos da classe IgM. A presença de anticorpos da classe IgM está relacionada à produção de autoanticorpos, não havendo contra-indicações ao transplante (DYER; MIDDLETON, 1993; KHODADADI et al., 2006).

Apesar do teste de CDC ser padrão "ouro" para realização da prova cruzada (*crossmatch*) pré-transplante, para a realização do teste de PRA, outras metodologias são utilizadas. Os testes de detecção de anticorpos anti-HLA têm-se aprimorado no sentido de aumentar a sensibilidade e especificidade dos resultados. Os testes sorológicos evoluíram, e passaram a utilizar ensaios de fase sólida. Dentre as técnicas para detecção de anticorpos, anti-HLA, utilizadas atualmente, estão: ELISA (do inglês, *Enzyme Linked Immunosorbent Assay*) e citometria de fluxo (*Flow Citometry*). Os testes de citometria de fluxo possuem alta sensibilidade que independem da disponibilidade e viabilidade de células do doador. No entanto, trata-se de testes de alto custo e que podem não estar disponíveis em todos os centros transplantadores. Além disso, as diferentes metodologias e os níveis de sensibilidade geram muitas dúvidas sobre a relevância clínica dos anticorpos detectados por elas.

O teste de imunoensaio de fase sólida utilizando a tecnologia Luminex consiste na detecção (teste de rastreio) de anticorpos anti-HLA (IgG) no soro de pacientes por meio da tecnologia de citometria de fluxo. Para isto dispõe de microesferas (2 a 4 µm de diâmetro) revestidas com antígenos HLA purificados de classe I ou de classe II de acordo com antígenos que se deseja pesquisar. O soro teste é primeiramente incubado com as microesferas. O anticorpo HLA presente no soro teste se liga aos antígenos fixados nas microesferas e então são marcados com IgG de cabra anti-humano conjugado com R-ficoeritrina (PE). O analisador de fluxo detecta a emissão fluorescente de PE de cada microesfera, permitindo quase uma aquisição de dados em tempo real. O padrão de reação do soro teste é comparado à uma planilha de leitura lote-específico definindo a série de antígenos para determinar o PRA e a especificidade HLA (ONELAMBDA, 2012).

Diversos estudos demonstraram que a sensibilização pré-transplante aos antígenos HLA é um fator de risco conhecido para a falência do enxerto (BARAMA et al., 2000). Percentuais elevados de PRA, associados ou não, ao aumento de incompatibilidade HLA, entre doador e receptor, resultam em um impacto negativo na evolução do enxerto (BARAMA et al., 2000; BRYAN et al., 2000).

1.2.5. **TIPIFICAÇÃO HLA**

Os testes de tipificação HLA do doador e receptor também são importantes, na investigação pré-transplante, pois, o número de incompatibilidades HLA (ou seja, número de antígenos no doador diferentes do receptor) pode estar associado à menor sobrevida do enxerto (OPELZ; DÖHLER, 2007). A maior compatibilidade HLA, entre doador e receptor, favorece a tolerância do sistema imune, aumentando a sobrevida do enxerto renal, tanto em transplante intervivos, quanto em doador falecido (OPELZ; DOHLER, 2007).

Até a década de 1990, as tipificações HLA também eram realizadas por métodos sorológicos e necessitavam de células frescas viáveis e um *pool* de anti-soros. Atualmente, foram substituídas por biologia molecular, por serem mais específicos, distinguirem a homozigosidade e o material biológico utilizado ser mais estável (DNA) (GHODKE et al., 2005). Apenas a identificação dos alelos HLA-A, HLA-B e HLA-DRB1 são rotineiramente executadas nos testes de histocompatibilidade pré-transplante de órgãos sólidos (ABBAS et al., 2015).

1.3. **DOENÇA RENAL CRÔNICA E TRANSPLANTE RENAL**

A doença renal crônica (DRC) é uma importante causa de morbimortalidade, gerando enormes custos aos Sistemas Nacionais de Saúde (CHERCHIGLIA et al., 2010). Verifica-se, a cada ano, um significante aumento na incidência e prevalência da população em programa dialítico. O aumento progressivo nas taxas de incidência deve-se, em grande parte, em decorrência do incremento da prevalência de algumas enfermidades crônico degenerativas como a hipertensão arterial sistêmica e o diabetes *mellitus* (BOMMER, 2002; CHERCHIGLIA et al., 2010b).

O transplante renal é o tratamento de escolha para uma parcela significativa dos pacientes com DRC. Esta opção possibilita a melhoria da qualidade de vida e sobrevida desses pacientes (SAYEGH; CARPENTER et al., 2004). A Associação Brasileira de Transplante de Órgãos estima que existam mais de 19.800 pacientes ativos em lista de espera para o transplante renal no primeiro trimestre de 2016 (BRASIL-RBT, 2016).

Visando avaliar o grau de compatibilidade, entre doador e receptor, e minimizar o risco de rejeição, os candidatos ao transplante renal são submetidos a vários exames laboratoriais. Dentre estes exames estão a tipificação HLA (HLA-A, -B e -DR), a prova

cruzada (*crossmatch*) doador específico e a avaliação da resposta imune humoral aos antígenos HLA (anticorpos anti-HLA), por meio do teste de PRA.

Devido à complexidade e custos que envolvem o processo de transplante renal, todas as formas de minimizar os riscos de rejeição são fundamentais, para o sucesso desse procedimento, e obtenção de maior sobrevida do enxerto renal.

Por estar presente em praticamente todas as células do organismo, o HLA é considerado um potente marcador de sobrevida em transplantes, apresentando papel de destaque entre os sistemas biológicos envolvidos no processo de rejeição, sendo até hoje, foco de muitos estudos.

1.4. **JUSTIFICATIVA**

Hoje, no Brasil, milhares de pacientes se encontram na lista de espera por um transplante. Enquanto aguardam por um órgão, esses pacientes podem entrar em contato com diferentes moléculas HLA e desenvolver níveis variados de anticorpos contra estas moléculas. Historicamente, o teste para detectar anticorpos anti-HLA tem sido, o teste de citotoxicidade dependente de complemento (CDC). Recentemente, testes mais sensíveis para detecção destes anticorpos, tais como os ensaios de imunoadsorção enzimática e os ensaios baseados em citometria de fluxo, podem contribuir para um aumento no número de candidatos em lista em espera de um transplante de órgãos. Contudo, a relevância clínica dos anticorpos detectados por estas metodologias ainda não está totalmente elucidada. Embora testes mais sensíveis estejam sendo amplamente utilizados, CDC continua a ser uma alternativa para definir o nível de sensibilização do paciente, é essencial na prova cruzada doador específico para evitar a rejeição hiperaguda e na detecção dos isotipos dos anticorpos.

Quanto maior o percentual de sensibilização do paciente em relação à população, menor é a chance de encontrar um doador apropriado, uma vez que a produção prévia de anticorpos contra o doador leva a uma resposta de rejeição hiperaguda acarretando a perda do enxerto. O transplante renal em pacientes sensibilizados permanece um desafio importante em nível mundial. Somado a isso, a porcentagem de candidatos sensibilizados em lista de espera por um órgão torna-se cada vez mais alto, por esta razão existe uma procura crescente de estratégias para melhorar a chance de transplantação destes pacientes.

Alguns estudos demonstraram a existência de diferentes respostas imunes de acordo com os fenótipos HLA apresentados pelo receptor. Estudos em populações turcas avaliaram por meio de técnicas sorológicas de tifipificação HLA, associações entre o fenótipo HLA-A, -

B e -DR com maior ou menor risco de sensibilização HLA em candidatos ao transplante renal em fase terminal da doença renal crônica (DRC). Apesar da clara importância da utilização de métodos moleculares na tipificação HLA, existem poucos trabalhos correlacionando as frequências alélicas nos pacientes renais de nossa região com o desenvolvimento da resposta imune humoral aos antígenos HLA. Além disso, a influência do genótipo HLA-C e HLA-DQ na sensibilização ainda são incertos. Como as tipificações HLA-C, -DQA1 e -DQB1 não são rotineiramente executadas nos testes de histocompatibilidade pré-transplante de órgãos sólidos, muito pouco se sabe sobre os efeitos desses alelos HLA na sensibilização, especialmente na população brasileira.

Das três principais causas de sensibilização, gravidez e transplantes são considerados "inevitáveis", enquanto que a transfusão sanguínea é a única potencialmente modificável ou "evitável". O uso de transfusão sanguínea em potenciais receptores de transplante renal tem sido muito debatido. A principal fonte de sensibilização HLA na maioria dos pacientes do sexo masculino que aguardam transplante renal é a transfusão sanguínea. Por esta razão, na maioria dos países, as transfusões sanguíneas têm sido evitadas em potenciais receptores de transplante renal. Apesar da importância da transfusão em sensibilização HLA, muito pouco se sabe sobre este particular evento de sensibilização em candidatos ao transplante renal no Brasil. Desse modo, a avaliação das causas de sensibilização na fase pré-transplante e da resposta imune aos antígenos HLA poderá auxiliar a equipe transplantadora no cuidado ao pacientes renal crônico referente às imunizações, naturais e artificiais.

1.5. **OBJETIVOS**

1.5.1. **GERAL**

Analisar as frequências dos grupos alélicos HLA e a resposta imune humoral aos antígenos HLA em pacientes renais, candidatos ao transplante, da região Norte/Noroeste do Estado do Paraná, Brasil.

1.5.2. **ESPECÍFICOS**

- Determinar o percentual de reatividade contra painel (PRA) utilizando os testes de Citotoxicidade dependente de complemento (CDC) e Imunoensaio de fase sólida utilizando a tecnologia Luminex (IFS);
- Realizar um estudo comparativo de desempenho dos testes de CDC e IFS na detecção de anticorpos anti-HLA;

- Verificar a associação entre presença de anticorpos anti-HLA e fatores de sensibilização (gestação, transfusão sanguínea e transplante);
- Determinar as especificidades de anticorpos anti-HLA em pacientes com PRA positiva;
- Determinar as frequências dos grupos alélicos HLA classe I (HLA-A, -B e -C) e classe II (HLA-DRB1, -DQA1 e -DQB1);
- Verificar a influência dos grupos alélicos HLA classe I (HLA-A, -B e -C) e classe II (HLA-DRB1, -DQA1 e -DQB1) na resposta imune humoral aos antígenos HLA.

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1. CAPÍTULO II

Artigo 1: "Complement-Dependent Cytotoxicity (CDC) to Detect Anti-HLA Antibodies: Old but Gold".

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Complement-Dependent Cytotoxicity (CDC) to Detect Anti-HLA Antibodies: Old but Gold

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Background: The criterion (gold) standard to detect anti-human leukocyte antigen (HLA) antibodies is the complement-dependent cytotoxicity (CDC) assay. Recently, more sensitive methods have been used for the same purpose. *Methods:* This study analyzed 70 serum samples of patients with end-stage renal disease using CDC, CDC with the addition of anti-human globulin (CDC-AHG), CDC with the addition of dithiothreitol (CDC-DTT), and the recent solid-phase immunoassay (SPI; Labscreen PRA) to detect anti-HLA antibodies. *Results:* Mean percent panel reactive antibodies (PRA) detected by SPI was 37.5% (±34.2) higher than the values detected by the other methods. Comparative analyses revealed significant difference between CDC and CDC-AHG, and between CDC and SPI ($P < 0.0001$), but not between CDC-AHG and SPI ($P = 0.8026$). *Conclusion:* Although the CDC-AHG method is "old," its performance to detect anti-HLA antibodies in the samples analyzed was comparable to the SPI in the evaluation of percent class I PRA. J. Clin. Lab. Anal. 28:275–280, 2014. © 2014 Wiley Periodicals, Inc.

Key words: alloantibodies; HLA; CDC; solid-phase immunoassay; transplantation; end-stage renal disease; panel reactive antibodies

INTRODUCTION

Kidney transplant is the best option for the treatment of end-stage renal disease (1). Anti-human leukocyte antigen (HLA) antibodies in the serum of patients who should receive organ transplants are a sign of an important risk factor (2). The method to detect anti-HLA antibodies has historically been the complement-dependent cytotoxicity (CDC) assay, described by Terasaki and McClelland in 1964 (3). Later, CDC with the addition of anti-human globulin (CDC-AHG) or dithiothreitol (CDC-DTT) was developed to improve the classical CDC assay. The addition of AHG enhances the amplification of the cytotoxicity reaction promoted by complement-fixing antibodies found in low titers and increases CDC sensitivity (4). Also, serum adequately treated with DTT improves the detection of antibodies to immunoglobulin G (IgG) and immunoglobulin M (IgM) isotypes (5). The importance of such improved detection lies on the fact that IgG isotype antibodies are deleterious for transplants and lead to hyperacute rejection (6, 7) and IgM isotype antibodies may be associated with the production of autoantibodies, in which case the transplant is not contraindicated (8).

Recently, more sensitive assays to detect anti-HLA antibodies, such as enzyme-linked immunosorbent assays and flow cytometry based assays (9) have contributed to an increase in the number of patients in the waiting lists of organ transplantation, although the clinical relevance of these antibodies remains unclear (2).

Donor-specific antibodies (DSA) detected by cross-match testing using CDC may suggest a contraindication for the transplant (2), so that hyperacute rejection and immediate loss of the graft may be avoided (6, 7). However, DSA detected exclusively by means of more sensitive

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assays should be regarded as an additional risk factor and not necessarily a contraindication to transplant success (2, 10, 11).

Although more sensitive assays have been widely used, CDC remains an alternative to define the level of patient sensitization (12), in crossmatching with a specific donor to prevent hyperacute rejection (13, 14) and detect antibody isotypes (15).

This study evaluated the performance of the CDC assay in comparison with a more recent technology, a solid-phase immunoassay (SPI) using the commercial kit LS1PRA® (One Lambda, Inc., Canoga Park, CA) to evaluate percent class I panel reactive antibodies (PRA) in samples of sera of patients waiting for a kidney transplant.

MATERIALS AND METHODS

Study Subjects

Sera of patients with end-stage renal disease

The sera analyzed in this study were collected from patients with end-stage renal disease in a single hemodialysis center in the city of Maringá, state of Paraná, Southern Brazil. All patients enrolled in the center participated in this study ($n = 70$).

PRA detected by CDC

The sera were analyzed using a panel of mononucleated cells isolated from 33 healthy donors. All serum samples were tested with CDC, CDC-AHG, and CDC-DTT. Results were described as percentages; percent PRA was negative when equal to zero (0%), and positive when greater than zero.

Serum treatment with DTT

For CDC-DTT assays, patient sera had to be previously treated with a DTT solution (Ultra Pure™, Invitrogen™, Inc., Carlsbad, CA) at a final concentration of 50 mM. Serum and DTT were incubated for 30 min at 37°C and agitated every 10 min. After treatment, sera were placed in a (Terasaki plate, One Lambda, Inc., Canoga Park, California, USA) for the CDC assay.

T-cell panel

For the cell panel, 33 healthy individuals of known HLA typing for A and B locus antigens underwent collection of 8 ml of peripheral blood in a container with acid citrate dextrose (Vacutainer® ACD, Becton Dickinson Diagnostic System, Buenos Aires, Argentina) (Table 1). Cells were separated using immunomagnetic beads (Fluorobeads® -T, One Lambda, Inc.) according to the manufacturer's instructions.

TABLE 1. HLA Specificities Used for the Cell Panel in the CDC Assay

A locus	Frequency	B locus	Frequency	B locus	Frequency
A1	4	B7	9	B52	2
A2	14	B8	3	B53	1
A3	6	B13	1	B55	1
A11	2	B14	3	B57	1
A23	1	B15	4	B58	3
A24	9	B18	6	B60	1
A26	2	B27	2	B63	1
A28	2	B35	5	B70	1
A29	4	B37	1		
A30	6	B40	7		
A31	1	B41	1		
A32	2	B44	5		
A33	1	B48	1		
A68	2	B50	1		
A74	2	B51	3		

CDC, complement-dependent cytotoxicity assay; HLA, human leukocyte antigen.

Assays

CDC assay

Initially, 1 μl of cells at a concentration of 3×10^6 cells/ml were placed onto the Terasaki plates where patient sera had been previously distributed. After incubation at 22°C for 30 min, 5 μl of rabbit complement (Imunodiagnóstica—Produtos e Serviços em Imunologia Ltda, Curitiba, Brazil) was added, followed by incubation for another 90 min. Immediately after that, the complement was removed from the plate by flickering and 5 μl of a stain-quench agent (FluoroQuench™, One Lambda, Inc.) was added. The reaction was visualized under inverted fluorescence microscopy (IBE 2,000, Applied Biosystems, Foster City, CA) and a 10× objective lens.

Results were described using scores from 1 to 8. These scores were based on the International Histocompatibility Workshop described by Dyer and Middleton (16). The reaction was positive when scores were equal to or greater than 2.

CDC-AHG assay

After incubation of sera and cells, as in the CDC assay described above, samples were rinsed three times, and 15 μl of phosphate-buffered saline was added to each well (each rinse, 10-min incubation). After plate flickering, 1 μl AHG (Goat IgG Anti-Human Kappa, One Lambda, Inc.) and, 1 min later, 5 μl of rabbit complement (Imunodiagnóstica—Produtos e Serviços em Imunologia Ltda)

TABLE 2. HLA Specificities Used for the Cell Panel in the SPI Test (LS1PRA, batch #10)

A locus	Frequency	B locus	Frequency	B locus	Frequency
A1	9	B7	3	B57	4
A2	17	B8	3	B58	3
A3	6	B13	3	B59	2
A11	10	B18	3	B60	3
A23	5	B27	3	B61	3
A24	6	B35	3	B62	4
A25	3	B37	2	B63	3
A26	5	B39	3	B64	2
A29	5	B41	2	B65	3
A30	5	B42	3	B67	2
A31	3	B44	3	B71	3
A32	5	B45	2	B72	3
A33	4	B46	2	B73	2
A36	3	B47	2	B75	2
A66	3	B48	2	B76	1
A68	6	B49	3	B78	1
A69	2	B50	2	B81	2
A74	3	B51	4	B8201	1
A80	2	B52	3		
		B53	3		
		B54	3		
		B55	3		
		B56	3		

HLA, human leukocyte antigen; SPI: solid-phase immunoassay.
Source: One Lambda Inc. (17).

were added, followed by incubation at 22°C for 90 min. The addition of stain and reading followed the same procedures as for the CDC assay.

CDC-DTT assay

CDC-DTT followed the same CDC protocol, and the only difference was the use of sera previously treated with DTT.

PRA detected by SPI

A commercial kit (LS1PRA®, One Lambda, Inc.) was used according to the manufacturer's instructions to detect class I anti-HLA antibodies. The antigen panel used in this kit (17) is described in Table 2. Data were acquired using a LABScan™ 100 unit (One Lambda, Inc.) and analyzed using the HLA-Visual™ software.

As for the cut off point for definition of positivity in the SPI, reactions with median fluorescence intensity greater than or equal to 500 were considered positive.

Statistical Analysis

The McNemar, chi-square, and paired t-tests were used to compare and estimate statistical significance of results. The level of significance was set at 5% ($P < 0.05$).

TABLE 3. Comparison of Mean Differences Between CDC, CDC-AHG, and SPI

Differences	Mean	Standard deviation	t_{cal}	P
(CDC-AHG) − (CDC)	17.2	23.2	6.20	<0.0001
SPI − CDC	23.4	23.6	8.27	<0.0001
SPI − (CDC-AHG)	6.2	21.9	2.37	0.0208

CDC, complement-dependent cytotoxicity assay; CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; SPI, solid-phase immunoassay.

The efficiency of the methods used to detect anti-HLA antibodies was assessed using diagnostic tests and calculating sensitivity, specificity, positive predictive value, and negative predictive value, as well as false positive and false negative results.

Ethics

This study was approved by the Ethics in Research Committee of Universidade Estadual de Maringá (no. 212/2009), and all ethic procedures defined in Resolution 196/96 of the Brazilian National Health Council (18) were followed.

RESULTS

Analysis of PRA Detected by CDC, CDC-AHG, and SPI

The comparison of CDC, CDC-AHG, and SPI showed that, of the 70 serum samples, the highest mean positivity in detecting anti-HLA antibodies was found for the SPI test 37.5% (±34.2), and percent PRA ranged from 0% (minimum value) to 98% (maximum value). In CDC and CDC-AHG, mean positivity was 14% (±26.8) and 31.5% (±37.5), respectively, and percent PRA ranged from 0% to 100% for both techniques.

The comparison of median values between the three methods also revealed that, in the SPI method, half of the serum samples had percentages equal to or lower than 28%, and the most frequent percent PRA was 0% (mode). The third quartile of the assays used showed that 75% of the samples analyzed using SPI had a percent PRA equal to or lower than 73%. The analysis of the third quartile of the other techniques showed a value of 63% for CDC-AHG and 21% for CDC without AHG (data not shown).

In addition to descriptive statistics, which analyzed continuous variables, mean differences were also compared between the three methods (paired t-test). Based on these data, this study found that, at a level of significance of 5%, there was a statistically significant difference when the mean difference was compared between the three methods (Table 3).

TABLE 4. Classification of Two of the Methods Under Study: CDC and SPI

SPI/CDC	Positive	Negative	Total
Positive	25 (a)	25 (b)	50
Negative	1 (c)	19 (d)	20
Total	26	44	70

CDC, complement-dependent cytotoxicity assay; SPI, solid-phase immunoassay.

TABLE 5. . Classification of Two of the Methods Under Study: CDC-AHG and SPI

SPI/CDC-AHG	Positive	Negative	Total
Positive	42 (a)	8 (b)	50
Negative	7 (c)	13 (d)	20
Total	49	21	70

CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; SPI, solid-phase immunoassay.

The McNemar test revealed that percent PRA detected by CDC and SPI was significantly different at a level of significance of 5% ($P < 0.0001$), which suggests that probability of positive results using the SPI is greater and confirmed that this method has a higher sensitivity than CDC without any additional treatments.

The diagnostic test used in this comparison confirmed the efficiency of SPI in the detection of anti-HLA antibodies. Sensitivity, specificity, positive predictive value, negative predictive value, false positive, and false negative values for the SPI, when CDC was used as the gold standard, were 96.2%, 43.2%, 50.0%, 95.0%, 50.0%, and 5.0%, respectively.

Only one of the serum samples of the 70 patients under analysis had a positive result by CDC and a negative result by SPI, which indicates that it was a false-negative case according to the diagnostic tests (Table 4, position c); however, previous analyses suggested that this patient had IgM isotype antibodies, which were, therefore, not detected by SPI.

The comparative analysis of CDC-AHG and SPI using the McNemar test showed that, at the level of significance of 5%, there were no significant differences between them ($P = 0.8026$). Sensitivity, specificity, positive predictive value, negative predictive value, false-positive and false-negative values for the SPI, when CDC-AHG was used as the gold standard, were 85.7%, 61.9%, 84.0%, 65.0%, 16.0%, and 35.0%, respectively. Later, serum treatment with DTT revealed the presence of IgM isotype in seven serum samples (Table 5, position c) negative by SPI and positive by CDC-AHG.

In addition, 18 serum samples had a higher percent PRA by CDC-AHG than by SPI (Fig. 1).

PRA Detected by CDC After Treatment With DTT

Previous treatment with DTT and subsequent testing with CDC-AHG revealed that 7 of the 70 samples under analysis were positive for IgM isotype. These results were confirmed using the SPI, which detects only the IgG isotype (Fig. 1).

DISCUSSION

Kidney transplants should be avoided in the presence of alloreactive cytotoxic antibodies due to the risk of hyperacute rejection (6). For this reason, crossmatching using the CDC microlymphocytotoxicity method remains widely used, even after the development of more sensitive methods (12).

The purpose of adding AHG to CDC is to eliminate low-affinity antibodies, amplify the cytotoxicity reaction promoted by low titer complement-fixing antibodies, and detect nonfixing complement antibodies, which increases the sensitivity of the method (4). As expected, the results of our analyses confirmed data found in the literature (19).

When PRA was analyzed using CDC-AHG after serum treatment with DTT (CDC-DTT), seven samples, previously positive, were negative, which suggests that the isotype detected before treatment was an IgM isotype. These results were confirmed using SPI, which detects only the IgG isotype (Fig. 1).

The DTT reduction agent enables the identification of the antibody isotype present in the patient's serum (IgM/IgG) (16). According to some authors, the presence of IgM isotype antibodies is associated with the production of autoantibodies, and there is no contraindication for transplantation in this case (8, 16). In contrast, the presence of IgG isotype antibodies against the specific donor cells is deleterious and may lead to hyperacute rejection (6, 7).

Some studies showed the importance of detecting IgM isotype antibodies and its association with graft survival. Yeğin et al. (20) reported the case of a patient who had positive CDC results that turned negative after the treatment with DTT. The patient received a living donor kidney transplant, and rejected 6 months after the transplant. The same authors reported another case, in which there were two positive CDC results that, after treatment with DTT, turned negative. In this case, the graft was preserved.

Khodadadi et al. (8) found convincing evidence that most IgM isotype antibodies do not play an important role in acute rejection. According to their results, 5.6% of the patients with IgM positive and IgG negative in crossmatching and PRA are expected to have a successful kidney transplant.

The action of IgM isotype antibodies in graft survival remains controversial, and it is fundamental to describe

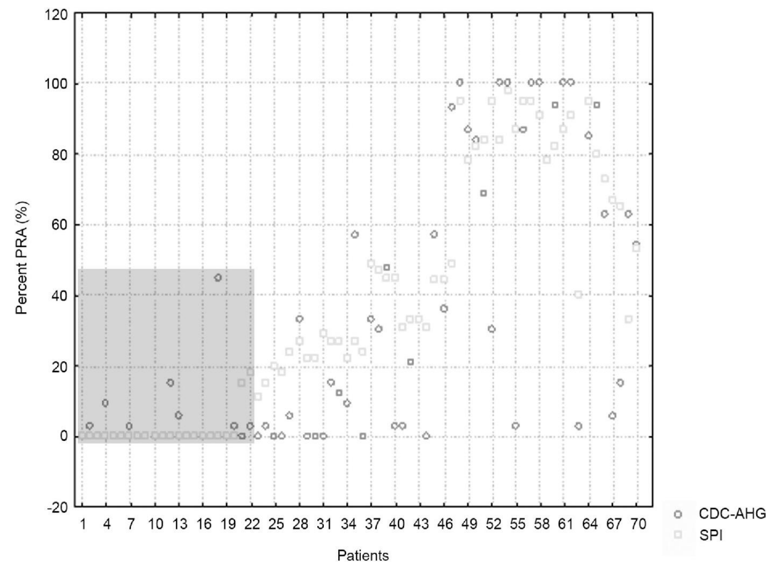

Fig. 1. Percent PRA detected by CDC-AHG and SPI. The highlighted area (gray) corresponds to seven patients with positive results by CDC and negative by SPI. CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; PRA, panel reactive antibodies; SPI, solid-phase immunoassay.

this class of antibodies using methods such as CDC-DTT. Moreover, patients who have this type of antibody detected before transplantation should be more carefully followed up.

The comparative analysis of CDC and SPI revealed a significant difference ($P < 0.0001$), which indicates a higher chance of positive results when using SPI and its greater efficiency in detecting anti-HLA antibodies. Sensitivity (96.2%) was in agreement with the study conducted by Colombo et al. (21), who found a high percent PRA when SPI was compared with CDC.

The comparison of CDC-AHG and SPI did not reveal any significant differences ($P = 0.8026$), which indicates that these methods have similar diagnostic performances. Although not evaluated statistically, PRA values for 18 of the 70 samples were higher by CDC-AHG than SPI (Fig. 1). This finding may probably be associated with the difference in frequency of HLA specificities used in each method (Tables 1 and 2).

Sensitivity (85.7%), evaluated according to the comparison between CDC-AHG and SPI, was lower than the value found in the comparison between CDC and SPI.

In general, percent PRA was higher by SPI than CDC-AHG (Fig. 1). However, Gibney et al. (22) reached the conclusion that, although percent PRA by SPI is higher, each individual should be examined carefully, because data found in their study pointed to the little importance of a high percent PRA in the absence of DSA when associated with cases of acute rejection or excellent graft survival. However, that same study suggested that, in the presence of DSA, SPI is a valuable test to identify possible clinical and immunological risks before transplantation.

Although current anti-HLA antibody detection methods are more sensitive and provide results in a shorter time, commercial kits do not identify isotypes (IgM/IgG) (15). In their review, Doxiadis, Roelen, and Claas (12) supported the use of the "old" CDC as the main method to define the level of sensitization and as an instrument to allocate kidneys for highly sensitized patients.

We understand that the CDC is a much more laborious method, which requires a panel of viable and available cells. However, this method has the advantage of reflecting a situation that more closely resembles the observed situation in vivo, because HLA, used as antibody targets, does not undergo manipulation.

We are also aware that problems involving cell viability, which include cell-based assays, have been solved with the introduction of solid-phase assays, since beads coated with HLA molecules are used as targets for antibody detection. However, based on our findings, we can state that, in order to detect percent PRA and distinguish between IgG and IgM antibodies, the CDC assay with the addition of AHG and DTT may still be an option in routine laboratory practice.

Undoubtedly, for the definition of anti-HLA antibody specificities, the SPI should be the method of choice. However, the purpose of this study was not to compare CDC assay sensitivity with a single-antigen assay, but rather

to the sensitivity of SPI-LS1PRA® (One Lambda, Inc.), which focuses more on sensitivity than on specificity in the detection of anti-HLA antibodies (23). We also performed this study to demonstrate that the CDC assay can continue to be used in routine laboratory procedures as a safe method to define the degree of sensitivity of patients on the waiting list for transplantation.

Of the limitations of our study, the small number of samples and the number of cells used to compose the T-cell panel for CDC were particularly relevant. Despite these limitations, data were enough for statistical analyses and purpose of this study.

CONCLUSION

Although CDC is an "old" method, the addition of AHG and the use of DTT led to the conclusion that its performance remains the criterion (gold) standard when compared with a more recent method (SPI-LS1PRA®, One Lambda, Inc.) to assess percent class I PRA and to define sensitized patients.

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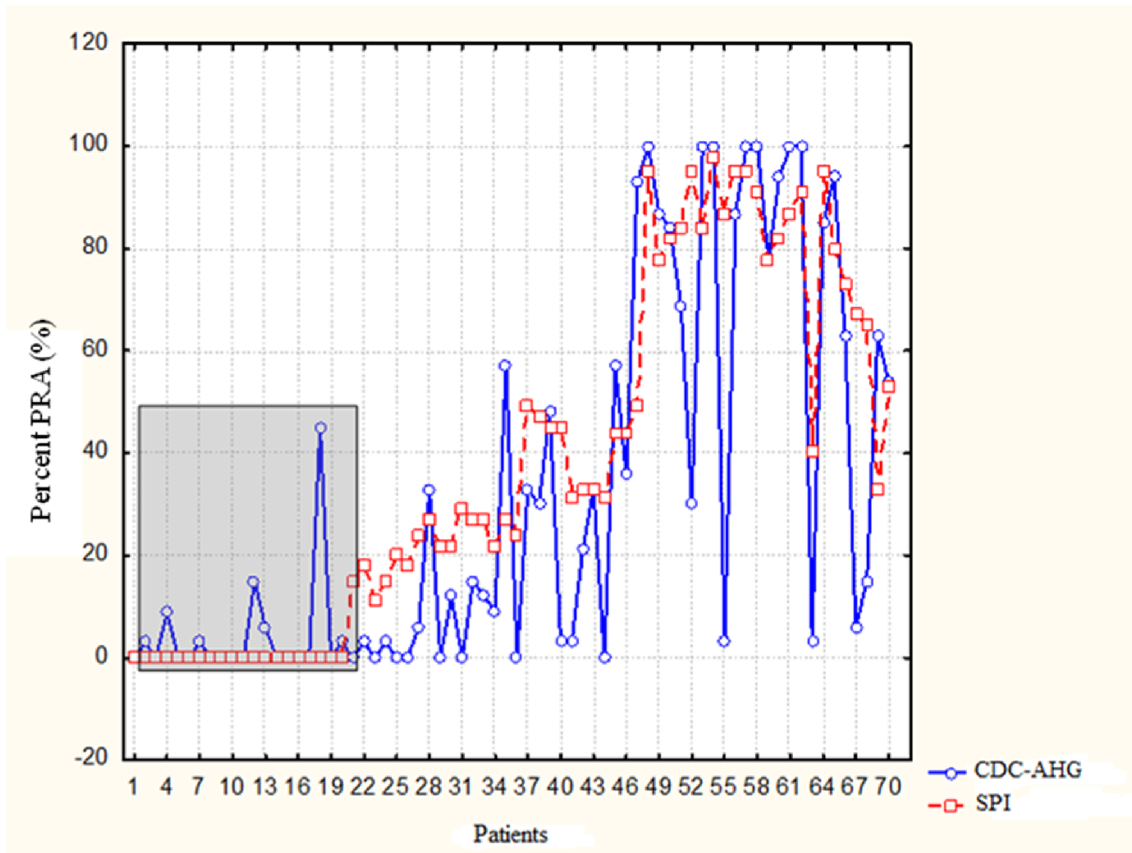

Material suplementar - Fig. 1 (Color Image). Percent PRA detected by CDC-AHG and SPI. The highlighted area (gray) corresponds to seven patients with positive results by CDC andnegative by SPI. CDC-AHG, complement-dependent cytotoxicity assay with the addition of anti-human globulin; PRA, panel reactive antibodies; SPI, solid-phase immunoassay.

Artigo 2: “HLA allele groups and decrease of humoral immune response to HLA antigens in Brazilian patients with chronic kidney disease”

HLA allele groups and decrease of humoral immune response to HLA antigens in Brazilian patients with chronic kidney disease

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ABSTRACT

Background: Pre-sensitization to human leukocyte antigen (HLA) is recognized as an important barrier to organ transplantation. However, the influence of HLA genotype on HLA sensitization has not been well reported. This study investigated the influence of HLA allele groups on humoral immune response to HLA antigens in Brazilian patients with chronic kidney disease (CKD). **Methods:** Sera from 319 patients, genotyped for HLA class I (HLA-A, -B, -C) and class II (HLA-DRB1, -DQA1, -DQB1), were screened by Luminex technology to detect anti-HLA class I and II antibodies. **Results:** Of the total patients, 63.6% had positive panel-reactive antibody (PRA). PRA-positivity was significantly associated with female gender ($p < 0.001$), transfusions ($p < 0.001$) and pregnancies ($p < 0.001$). The frequencies of *HLA-B*14* (OR: 3.32; CI: 1.13-9.76), *HLA-C*08* (OR: 3.98; CI: 1.38-12.38) and *HLA-DRB1*16* (OR: 3.32; CI: 1.13-9.76) were significantly higher in patients with negative PRA compared with patients with positive PRA, suggesting that these HLA allele groups might be involved in the decrease of humoral immune response to HLA antigens. **Conclusion:** This study confirmed the significant association between female gender, transfusions and pregnancies with PRA-positivity and suggested the involvement of HLA allele groups in the decrease of anti-HLA antibody formation.

Key-words: Antibodies; Kidney failure, chronic; Kidney transplantation; HLA antigens; Gene frequency.

INTRODUCTION

Anti-human leukocyte antigen (HLA) antibodies are recognized as an important barrier to organ transplantation, since they are directly related to graft failure [1]. The production of these antibodies occurs mainly after transfusion of blood products [2, 3], pregnancy [3-5] and transplantation [3, 6].

The presence of preformed anti-HLA antibodies is a known risk factor for acute rejections and allograft failure [1, 3]. These antibodies can be detected by panel-reactive antibody (PRA) test. Patients with high percentages of PRA have many difficulties to find a crossmatch-negative donor for transplantation and increased risk of hyperacute/acute graft failure and graft dysfunction [7, 8].

The patient immune response level varies from person to person and is related to HLA system. Due to the HLA molecules are glycoproteins present on the cell surface, involved in the recognition and presentation of many epitopes [9], studies demonstrated that the HLA typing of the recipient might influence the production of anti-HLA antibodies [10, 11].

Studies in Turkish population evaluated associations of HLA-A, -B and -DR phenotype with increased or decreased risk of HLA sensitization in end-stage renal disease (ESRD) candidates for kidney transplantation [10, 11]. However, the influence of HLA-C and -DQ genotype on HLA sensitization is still uncertain. As routinely HLA-C , -DQA1 and -DQB1 typifications are not performed in histocompatibility testing for solid organ transplantation, very few studies evaluated the effects of these alleles on HLA sensitization.

This study investigated the influence of HLA class I (HLA-A, -B, -C) and class II (HLA-DRB1, -DQA1, -DQB1) allele groups on humoral immune response to HLA antigens in Brazilian patients with chronic kidney disease (CKD) based in sensitizing events.

MATERIALS AND METHODS

Patients

The current study included 319 patients (mean age: 47.23 years), with CKD, renal transplant candidates, who were registered in Regional Transplant Centers from northern and northwestern Paraná State, Brazil, in March 2011. Only patients with updated records (active patients/potential recipients) were included. Information on the demographic characteristics

and sensitizing events (transfusions, pregnancies and previous transplants) were reported by the transplant center and dialysis clinics.

To compare the demographic characteristics and occurrence of sensitizing events, the patients were divided into two groups, according to the PRA results:

PRA-negative group: PRA = 0 (n = 116).

PRA-positive group: PRA > 0 (n = 203).

Subsequently, to compare the frequencies of HLA genotypes, the patients were divided into two groups, according to the PRA results and occurrence of any sensitizing events:

Group 1: patients who had sensitizing events and negative PRA (n = 73).

Group 2: patients who had sensitizing events and positive PRA (n = 175).

DNA extraction and HLA genotyping

To perform the HLA genotyping, 5 mL of blood of the patients was collected by venipuncture into vacuum tubes (Vacutainer, Becton and Dickson, Oxford, UK) containing ethylene diamine tetraacetic acid (EDTA) as anticoagulant. Genomic DNA was extracted by the separation column method, using the commercial kit for DNA extraction Biopur (Biometrix, Curitiba, Paraná, Brazil), following the manufacturer's protocol. After adjusting the DNA concentration, obtained by the optical density method, DNA was amplified using polymerase chain reaction-sequence specific primers (PCR-SSO) combined with Luminex technology. Genomic DNA was amplified using biotinylated sequence-specific primers for HLA class I (HLA-A, -B, -C) and class II (HLA-DRB1, -DQA1, -DQB1) in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Following by hybridization with complementary probes for DNA, conjugated with microspheres (beads) labeled with different fluorochromes to identify complementary sequences of the amplified DNA, using the commercial kit LABType (One Lambda, Inc., Canoga Park, CA, USA), following the manufacturer's protocol. After hybridization, the results were read using the flow cytometry platform LABScan^{TM}100 (One Lambda, Inc.), followed by analysis using the program HLA Fusion version 2.0 (One Lambda, Inc.).

Determination of percentages of panel-reactive antibodies (PRA)

The percentages of PRA were determined in sera of patients, using the LS1PRA and LS2PRA commercial kits (One Lambda, Inc.) combined with the Luminex technology, following the manufacturer's protocol. After processing, the samples were read with the flow cytometry platform LABScan™100 (One Lambda, Inc.), followed by analysis in HLA Fusion version 2.0 software (One Lambda, Inc.).

Statistical analyses

Statistical analyses were performed using Statistica 7.0 and R programs. Allele frequencies were calculated by direct counting. Fisher's exact test and Student's T test were used to compare the demographic characteristics and sensitizing events among the study groups. Significant differences between the allelic frequencies in Group 1 and in Group 2 were calculated using the Fisher's exact test with mid p adjustment. *P*-values of less than 0.05 were considered statistically significant. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to evaluate the risk association.

RESULTS

Demographic characteristics and sensitizing events of the patients according to PRA results are shown in Table 1.

The PRA-positive group consisted of 203 patients (63.6%), and the PRA-negative group of 116 (36.4%) patients. PRA-positivity was significantly associated with female gender ($p < 0.001$), transfusions ($p < 0.001$) and pregnancies ($p < 0.001$) (Table 1).

A total of 20 HLA-A, 30 HLA-B and 14 HLA-C allele groups in HLA class I were identified. The *HLA-A*02, A*24, A*01, B*35, B*44, C*07, C*04* and *C*03* allele groups were found with frequencies greater than 10% in the total sample (Table 2).

A total of 13 HLA-DRB1, 6 HLA-DQA1 and 5 HLA-DQB1 allele groups in HLA class II were identified. The *HLA-DRB1*11, DRB1*04, DRB1*13, DRB1*07, DRB1*03, DQA1*01, DQA1*05, DQA1*03, DQA1*02, DQB1*03, DQB1*06, DQB1*05* and *DQB1*02* allele groups were found with frequencies greater than 10% in the total sample (Table 3).

Among the total patients, there were 43 who not having sensitizing event history and had negative PRA. Furthermore, 73 patients had sensitizing events but not developed any

anti-HLA antibodies and 28 patients had anti-HLA antibodies although not having any sensitizing event history.

When the HLA allele frequencies were evaluated in Group 1 (patients who had sensitizing events and negative PRA; n = 73) and Group 2 (patients who had sensitizing events and positive PRA; n = 175), *HLA-B*14* (p = 0.033; OR: 3.32; CI: 1.13-9.76), *HLA-C*08* (p = 0.006, OR: 3.98; CI: 1.38-12.38) and *HLA-DRB1*16* (p = 0.033; OR: 3.32; CI: 1.13-9.76) were significantly high in Group 1 (Table 2 and 3).

DISCUSION

The relevance of HLA-A, -B, and -DR has been known since early years of clinical organ transplantation [7], while the importance of HLA-C and -DQ has recently been documented [12, 13]. Previous studies analyzed possible associations of HLA-A, -B and -DR phenotype with anti-HLA antibody status and have suggested that certain HLA phenotype may influence the development of anti-HLA antibodies [10, 11]. To our knowledge, this study is the first study investigating the HLA genotype (especially, -C and -DQ) and HLA sensitization in South American patients with CKD.

It is well known that sensitization to HLA antigens usually can occur through exposure to HLA alloantigens such as blood products, foreign tissue during transplantation or in females, during pregnancies [2-6]. In concordance with the literature, results of current study confirmed the significant association of PRA-positivity with female gender, pregnancies and blood transfusions [10, 11, 14, 15].

Our study identified 73 patients who had one or more sensitizing events but not developed any anti-HLA antibodies and 28 patients who had anti-HLA antibodies although not having any known sensitizing event history. Class and van Rood (1988) [16] demonstrated that not every patient had the same probability to become sensitized after sensitizing events. Some individuals with HLA alloantigen exposure do not produce the expected antibodies, the reason for this, might involve many factors such as immunogenicity of the product, altered state of immune responsiveness or immune response gene regulation [10].

Despite sensitizing events, anti-HLA antibodies can also, although rare, develop “spontaneously”. This could be due to cross-reactive microbial determinants [17] and pro-inflammatory events [18]. Spontaneous development of anti-HLA antibodies have been

detected in patients without any known sensitizing events, these "spontaneous" anti-HLA antibodies are likely non-specific and arise from an upregulation of the immune system from pro-inflammatory events or cross-sensitization following infections, such as hepatitis C and influenza [19-22].

The Brazilian population is characterized by a wide mix of ethnic groups, a result of immigration from several countries during the colonization of Brazil, over a period of five centuries, resulting in an interracial mixture of Europeans, Africans, Amerindians and Asians [23]. The samples evaluated in our study are from the Paraná State, in Southern Brazil. This state was colonized largely by Europeans, and now has a high proportion of Caucasians. However, persons of others ethnic groups are also frequent in the population [24]. These influences can be observed in the results of the current study, which show a high contribution of HLA alleles of European origin, such as *HLA-A*02*, *-B*44, -C*04, -DRB1*11, -DRB1*04, -DQA1*05* and *-DQB1*03* [25, 26] as well as the occurrence of others alleles of various ethnic origins.

The general distribution of the most common HLA-A, -B, and -DRB1 allele groups is similar to that found in other studies in Southern Brazil, including Paraná State [27, 28], where the health populations studied were also described as mostly Caucasian [27-29].

Due to biological function and high degree of polymorphism, the HLA system has been studied as a genetic marker involved in susceptibility to different diseases [30, 31]. Studies in Turkish population have shown associations between HLA phenotypes and HLA antibodies status [10, 11]. Current study demonstrated that the frequency of *HLA-B*14, HLA-C*08* and *HLA-DRB1*16* was significantly high in Group 1 (patients who did not develop any anti-HLA antibodies, although they have been exposed to foreign HLA) compared with Group 2 (patients who had sensitizing events and developed anti-HLA antibodies), suggesting that these HLA allele groups might be associated with decrease of humoral immune response to HLA antigens. However, the immune response gene control and genetic factors responsible for anti-HLA antibody formation are not entirely clarified; probably involve a regulation through polymorphic HLA molecules in their physiologic roles of antigen processing and presentation to the immune cells [10].

Studies of association between HLA phenotypes and anti-HLA antibodies status have shown different results. Heise et al. (2001) [10] concluded in univariate analysis of the entire cohort that nine HLA allelotypes (DR1,4,7; B8,12,40; A1,2,11) were associated with a significantly decreased risk of HLA sensitization. Results of Karahan et al. (2010) [11]

demonstrated that *DRB1*07* may be associated with low risk of anti-HLA antibodies development.

In study of HLA polymorphisms as incidence factor in the progression to ESRD in Brazilian patients awaiting kidney transplant the HLA-B14 was negatively associated with CKD, this phenotype had a significantly lower frequency among patients compared with controls [31].

The majority studies mentioned above utilized serologic typing, which can have drawback of resolution, accuracy and reproducibility [32]. Furthermore, due to differences in the frequencies of HLA alleles among ethnic groups, some associations might be found in certain populations and not in others [33]. As the Brazilian population is one of the most heterogeneous in the world, variations in the frequencies of HLA alleles might be found according to the predominant ethnic group in each region [24, 29, 34]. For this reason, was difficult to compare our patients group with others studies conducted in different countries.

Some limitations of this study include the sample size; however, this number represents approximately 32% and 12% of the active renal transplant candidates registered in the waiting list in Paraná state and in Southern Brazil, respectively [35]; and the difficulties to obtain relevant information about sensitizing events, because the majority of dialysis clinics and transplant centers in Brazil do not have an informatics system for efficient data storage, updating and retrieval of this information. Despite these limitations, this study provides important data on HLA polymorphism and sensitization in Brazilian patients with CKD.

CONCLUSIONS

This study confirmed the significant association between female gender, transfusions and pregnancies with PRA-positivity. The HLA class I (*HLA-B*14, -C*08*) and class II (*HLA-DRB1*16*) allele groups might be involved in the decrease of humoral immune response to HLA antigens in Brazilian patients with CKD. Future studies encompassing larger group of patients will need to assess the clinical relevance of the results obtained and should contribute to the elucidation of genetic markers involved in the regulation of humoral immune response to HLA antigens in patients with CKD.

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COMPLIANCE WITH ETHICAL STANDARDS

Disclosure: All the authors have declared no competing interest.
Ethical approval: This study was approved by the Ethics Committee of the Universidade Estadual de Maringá, Paraná, Brazil (protocol no. 333/2011). All procedures followed Resolution 466/2012 of the Brazilian Health Council which rules on research work on humans.
Informed consent: Informed consent was obtained from all individual participants included in the study.

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TABLES

Table 1. Demographic characteristics and sensitizing events, according to the Panel-reactive antibody (PRA) results.

	PRA - Positive (n=203)	**PRA - Negative (n=116)**	***p* - value**
Mean age (years)	47.7 ± 13.1	46.4 ± 12.2	0.384
Gender			<0.001
Males	111 (54.7%)	87 (75.0%)	
Females	92 (45.3%)	29 (25.0%)	
Ethnic group			0.199
Caucasians	105 (51.7%)	68 (58.6%)	
Mestizos	62 (30.5%)	27 (23.3%)	
Blacks	33 (16.3%)	16 (13.8%)	
Orientals	3 (1.5%)	5 (4.3%)	
Sensitizing events			
Any transfusions (nº of patients)	153 (75.4%)	47 (40.5%)	<0.001
Any pregnancies (nº of females)	75 (81.5%)	12 (41.4%)	<0.001
Previous transplantation history (nº of patients)	19 (9.4%)	4 (4.3%)	0.124

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Table 2. HLA class I (-A, -B and -C) allele groups frequencies in the total samples and their comparisons between study groups.

	Total	Group 1	Group 2	Group 1 x Group 2	
HLA	f a	f a	f a	*p* value	OR(CI95%)
*A*01*	0.1018	0.0890	0.0914	1.000	
*A*02*	0.2382	0.2465	0.2485	1.000	
*A*03*	0.0940	0.0958	0.1000	1.000	
*A*11*	0.0595	0.0479	0.0685	0.542	
*A*23*	0.0454	0.0342	0.0514	0.489	
*A*24*	0.1034	0.1301	0.0800	0.093	
*A*25*	0.0282	0.0342	0.0342	1.000	
*A*26*	0.0485	0.0410	0.0628	0.399	
*A*29*	0.0360	0.0273	0.0314	1.000	
*A*30*	0.0579	0.0890	0.0571	0.235	
*A*31*	0.0329	0.0342	0.0285	0.776	
*A*32*	0.0250	0.0205	0.0285	0.764	
*A*33*	0.0297	0.0479	0.0257	0.263	
*A*34*	0.0094	0.0136	0.0114	1.000	
*A*36*	0.0047	0.0068	0.0028	0.502	
*A*66*	0.0078	0.0000	0.0028	1.000	
*A*68*	0.0532	0.0273	0.0485	0.338	
*A*69*	0,0015	0.0000	0.0028	1.000	
*A*74*	0.0188	0.0136	0.0171	1.000	
*A*80*	0.0031	0.0000	0,0057	1.000	
*B*07*	0.0595	0I0547	0.0657	0.839	
*B*08*	0.0564	0.0410	0.0457	1.000	
*B*13*	0.0172	0.0205	0.0142	0.698	
*B*14*	0.0266	0.0547	0.0171	0.033	3.32 (1.13-9.76)
*B*15*	0.0940	0.0890	0.1142	0.524	
*B*18*	0.0423	0.0547	0.0314	0.211	
*B*27*	0.0282	0.0205	0.0228	1.000	
*B*35*	0.1097	0.0890	0.1142	0.524	
*B*37*	0.0078	0.0068	0.0057	1.000	
*B*38*	0.0297	0.0342	0.0314	1.000	

*B*39*	0.0344	0.0342	0.0400	1.000	
*B*40*	0.0579	0.0684	0.0600	0.689	
*B*41*	0.0188	0.0136	0.0171	1.000	
*B*42*	0.0235	0.0205	0.,0257	1.000	
*B*44*	0.1097	0.1027	0.1142	0.756	
*B*45*	0.0188	0.0273	0.0171	0.490	
*B*47*	0.0015	0.0000	0.0000	-	
*B*48*	0.0062	0.0000	0.0057	1.000	
*B*49*	0.0329	0.0342	0.0342	1.000	
*B*50*	0.0297	0.0136	0.0314	0.362	
*B*51*	0.0846	0.0753	0.0885	0.725	
*B*52*	0.0156	0,0273	0.0142	0.459	
*B*53*	0.0266	0.0136	0.0257	0.521	
*B*54*	0.0031	0.0068	0.0028	0.502	
*B*55*	0.0109	0.0136	0.0114	1.000	
*B*56*	0,0015	0.0000	0.0028	1.000	
*B*57*	0.0313	0.0410	0.0285	0.577	
*B*58*	0.0172	0.0342	0.0142	0.168	
*B*81*	0.0015	0.0000	0.0028	1.000	
*B*82*	0,0015	0.0068	0.0000	0.294	
*C*01*	0.0297	0.0205	0.0314	0.767	
*C*02*	0.0736	0.0616	0.0828	0.465	
*C*03*	0.1034	0.1095	0.1057	0.875	
*C*04*	0.1536	0.1164	0.1657	0.172	
*C*05*	0.0454	0.0342	0.0457	0.635	
*C*06*	0,0971	0,0958	0.0942	1.000	
*C*07*	0.2147	0,2054	0.2142	0.904	
*C*08*	0.0329	0.0753	0.0200	0.006	3.98 (1.38-12.38)
*C*12*	0.0877	0.1164	0.0800	0.229	
*C*14*	0.0250	0.0205	0.0285	0.764	
*C*15*	0.0470	0.0410	0.0514	0.818	
*C*16*	0.0470	0.0547	0.0371	0.463	
*C*17*	0.0391	0.0410	0.0428	1.000	

*C*18*	0.0031	0.0068	0,0000	0.294	

fa, allelic frequencies; Group 1 (patients who had sensitizing events and negative PRA; n = 73); Group 2 (patients who had sensitizing events and positive PRA; n = 175); OR; Odds ratio; CI, Confidence Interval.

Table 3. HLA class II (-DRB1, -DQA1 and -DQB1) allele groups frequencies in the total samples and their comparisons between study groups.

	Total	Group 1	Group 2	Group 1 x Group 2	
HLA	f a	f a	f a	*p* value	OR(CI95%)
*DRB1*01*	0.0940	0.0821	0.0971	0.734	
*DRB1*03*	0.1050	0.1095	0.0971	0,744	
*DRB1*04*	0.1347	0.1027	0.1485	0,196	
*DRB1*07*	0.1112	0.1301	0.1114	0.543	
*DRB1*08*	0.0564	0.0273	0.0657	0.126	
*DRB1*09*	0.0188	0.0205	0.0142	0.698	
*DRB1*10*	0.0235	0.0273	0.0257	1,00	
*DRB1*11*	0.1394	0.1643	0.1285	0.319	
*DRB1*12*	0.0125	0.0136	0.0114	1.00	
*DRB1*13*	0.1222	0.1164	0.1200	1.00	
*DRB1*14*	0.0517	0.0547	0.0514	0.829	
*DRB1*15*	0.0971	0.0958	0.1114	0.749	
*DRB1*16*	0.0329	0.0547	0.0171	0.033	3.32 (1.13-9.76)
*DQA1*01*	0.4090	0.4520	0.3971	0.272	
*DQA1*02*	0.1222	0.1575	0.1228	0.312	
*DQA1*03*	0.1504	0.1164	0.1600	0.266	
*DQA1*04*	0.0564	0.0342	0.0628	0.277	
*DQA1*05*	0.2539	0.2397	0.2428	1.000	
*DQA1*06*	0.0078	0.0000	0.0142	0.328	
*DQB1*02*	0.1912	0.1849	0.1942	0.900	
*DQB1*03*	0.3354	0.3150	0.3371	0.676	
*DQB1*04*	0.0689	0.0616	0.0742	0.703	
*DQB1*05*	0,2006	0.2465	0.1857	0.142	
*DQB1*06*	0.2037	0.1917	0.2085	0.715	

fa, allelic frequencies; Group 1 (patients who had sensitizing events and negative PRA; n = 73); Group 2 (patients who had sensitizing events and positive PRA; n = 175); OR, Odds ratio; CI, Confidence Interval.

Artigo 3: "Sensitization to human leukocyte antigen (HLA) and transfusion in male Brazilian patients awaiting primary kidney transplant".

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Sensitization to human leukocyte antigen (HLA) and transfusion in male Brazilian patients awaiting primary kidney transplant

Short title: HLA antibodies and transfusion

Summary

Background: Currently, the main source of HLA sensitization in most male patients awaiting primary kidney transplant will be blood transfusion. Despite the known importance of the transfusion in HLA sensitization, very little is known about of this particular sensitization event in Brazilian renal transplant candidates. **Methods:** This study evaluated the sensitization to HLA antigens and transfusion in 236 male Brazilian patients awaiting their first kidney transplant. Panel-reactive antibody (PRA) was performed by LS1PRA/LS2PRA commercial kits (One Lambda, Inc.). **Results:** Of the 236 patients, 121 (51.3%) had positive PRA (PRA>0) and 138 (58.5%) had previous history of blood transfusion. Transfusion history showed a significant difference between the PRA-positive and PRA-negative group ($p<0.001$). Sensitization from transfusion occurred in 88 (37.3%) patients. Transfused patients had longer waiting times on dialysis when compared with nontransfused patients ($p<0.01$). PRA class I or/and class II positive and median number of HLA class I or/and class II specific antibody was higher for those who received transfusion compared to those who did not received transfusion ($p<0.05$). **Conclusion:** This study document a high proportion of sensitization (positive PRA) and transfusion history in this population, indicating that many candidates continue to receive blood transfusions before transplantation and become sensitized.

Key-words: Antibodies; Blood transfusion; Kidney failure, chronic; Kidney transplantation; HLA antigens.

Introduction

Human leukocyte antigen (HLA) sensitization represents an important barrier to successful kidney transplantation. The presence of donor-specific circulating anti-HLA antibodies in pre-transplant stage has generally been associated with an increased risk for hyperacute rejection and poor long-term allograft survival [1, 2]. Patients can become sensitized mainly after exposure to foreign HLA antigens through blood transfusions [3], pregnancy [4-6] and organ transplantation [6, 7].

Kidney transplantation in HLA-sensitized patients remains an important challenge worldwide. Furthermore, the percentage of HLA-sensitized patients in waiting list for an organ becomes increasingly high, for this reason there is a growing demand for strategies to enhance transplantation of these patients. Of the three principal causes of sensitization, pregnancies and transplants are considered unavoidable, while blood transfusion is the only potentially modifiable, medically 'preventable' [8].

The use of blood transfusion in potential renal transplant recipients has long been debated. The main source of HLA sensitization in most male patients awaiting primary kidney transplant will be blood transfusion [9]. For this reason, in most countries, whenever possible, blood transfusions have been avoided in potential renal transplant recipients [10]. The decision to transfuse should always be based on a careful assessment of the risks and benefits to the patient, balancing the risk of HLA sensitization against the clinical need to save life or prevent significant morbidity [9-11].

Despite the importance of the transfusion in HLA sensitization, very little is known about of this particular sensitization event in Brazilian renal transplant candidates. Recent studies in United States population demonstrated that sensitization from transfusion still can occur in many renal transplant candidates, resulting in higher antibody levels [8, 12]. Thus, the HLA sensitizing effect of blood transfusion combined with other potential adverse effect adds further evidence in favor of restrictive transfusion strategies in potential transplant recipients [8, 13]. In an effort to inform and to help in clinical decision making with regards to transfusion in patients awaiting transplantation, this study evaluated the sensitization to HLA antigens and transfusion in male Brazilian patients awaiting primary kidney transplant.

Materials and methods

Study Population

The study population consisted of 236 Brazilian male patients with chronic renal failure, undergoing hemodialysis therapy, candidates for a primary transplant, who were registered in kidney transplantation waiting list, in June 2010. Only patients with updated records (active patients/potential recipients) were included. Data regarding demographic characteristics and previous history of blood transfusions were reported by the transplant centers and dialysis clinics.

According to the results of the percentage of panel-reactive antibodies (PRA), the study population was divided into two groups: PRA-negative (PRA = 0) and PRA-positive (PRA > 0). Subsequently, according to the transfusion history, the same patients were divided into two groups: transfused and nontransfused. The first group included patients who did not have previous history of blood transfusions, and the second group included patients who had one or more blood transfusions episodes.

This study was approved by the Ethics Committee of the Universidade Estadual de Maringá, Paraná, Brazil (protocol no. 333/2011). All procedures followed Resolution 466/2012 of the Brazilian Health Council, which rules on research work on humans in Brazil. All procedures were explained to each subject, and written informed consent was obtained from each subject.

Determination of percentages of panel-reactive antibodies (PRA) and HLA-specific antibodies

The percentages of PRA and HLA class I and class II specific antibody were determined in sera from the patients, using the LS1PRA and LS2PRA commercial kits (One Lambda, Inc.) combined with the Luminex technology, following the manufacturer's protocol. After processing, the samples were read with the flow cytometry platform LABScan^{TM}100 (One Lambda, Inc.), followed by analysis in HLA Fusion version 2.0 software (One Lambda, Inc.) using the fluorescence analysis minimum recommended by the manufacturer (median fluorescence intensity equal to or greater than 500).

Statistical analyses

All statistical analyses were performed using Statistica 7 software. Continuous data were described with the use of mean and standard deviation (SD), and categoric data were expressed as n (%). Antibody frequencies were calculated by direct counting. Fisher's exact

test and Student's T test were used to compare the demographic characteristics, transfusion history and anti-HLA antibody status among the study groups. The significance level of the statistical test was 5% ($p < 0.05$).

Results

Of the 236 patients included into the study, 138 (58.5%) patients had previous history of blood transfusion. The demographic characteristics and transfusion history, according to the PRA results, are shown in Table 1.

The PRA-positive group consisted of 121 (51.3%) patients, and the PRA-negative group of 115 (48.7%) patients. Only transfusion history showed a significant difference between the two groups ($p < 0.001$).

Among the 121 patients with positive PRA, 86 (71.1%) were class I positive, 68 (56.2%) were class II positive and 33 (27.3 %) were both class I and II positive.

The overall incidence of anti-HLA-A, -B, -C, -DR, -DQ and -DP antibodies in the group of patients with positive PRA is shown in Tables 2 and 3. The most frequent specificities of anti-HLA antibodies were: anti-A34, B27, C16, DR12, DQ8, DP3, DP13 and DP18.

The demographic characteristics and PRA results, according to the transfusion status, are shown in Table 4.

Sensitization to HLA antigens from transfusion occurred in 88 (37.3%) patients.

Dialysis duration showed a significant difference between transfused and nontransfused patients ($p < 0.01$). Transfused patients had longer waiting times in dialysis when compared with nontransfused patients.

Overall; PRA class I or/and class II positive showed a significant difference between transfused and nontransfused patients (class I or class II, $p < 0.001$; class I, $p < 0.001$; class II, $p < 0.01$; class I and II, $p = 0.013$). PRA positive was higher in transfused patients. Furthermore, median number of HLA class I or/and class II specific antibody were higher for those who received transfusion compared to those who did not received transfusion (class I, $p < 0.01$; class II, $p < 0.001$; class I and II, $p < 0.001$).

Discussion

In a scenario where deceased donor organs are scarce, waiting times for transplantation are long and kidney transplantation in sensitized patients remains a significant problem worldwide, the HLA sensitizing effect of blood transfusion adds further evidence in favor of transfusion-minimizing strategies in all persons who may eventually require kidney transplantation in the future. To our knowledge, this study provides the first data on the sensitization to HLA antigens (PRA and HLA specific antibody) from transfusion in male South American patients awaiting primary kidney transplant.

It is well known that HLA sensitization is caused mainly by previous blood transfusion, pregnancy or solid organ transplantation [3-7]. For this reason, current study analyzed male patients awaiting primary kidney transplant to avoid confounding sensitization event.

The vast majority of patients investigated in the present study received blood transfusions (58.5%). Blood transfusion remains an important risk factor for HLA sensitization in patients in hemodialysis treatment. Although the use of recombinant human erythropoietin in dialysis patients has resulted in improved hemoglobin levels and have substantially decreased the need for red blood cell transfusions, its use does not completely eliminate the need for blood transfusions in renal patients, mainly due to increasing age and cardiovascular comorbidities in these patients [9, 13-15].

Our study documented the frequency of anti-HLA antibodies in male Brazilian patients awaiting primary kidney transplant, specially, anti-HLA-C and anti-HLA-DP antibodies, which has not been well documented in Brazilian studies. The presence of anti-HLA-C and anti-HLA-DP has been reported in hyperacute or acute rejection [16-21] and antibody-mediated chronic rejection [22, 23] in renal transplant recipients. These findings emphasize the importance of knowledge of these antibodies, in order to improve graft survival in the post-transplantation.

Patients who received transfusion had longer waiting times on dialysis when compared with those who did not received transfusion. Sensitized patients have longer waiting times at the transplant waiting list for finding compatible donors, remain longer times in dialysis treatment and therefore may develop relevant increase of anti-HLA antibodies by different sensitizing events, including blood transfusions [12, 24].

The transfusion history was associated with HLA sensitization. Additionally, the number of HLA-specific antibody and PRA levels was higher in transfused patients than in nontransfused patients. Recent studies provided evidence for the HLA sensitizing effect of

transfusions [8, 12, 24]. Transfusion of blood and blood components exposes the patient to HLA alloantigens expressed on the surface of donor red blood cells, leukocytes and platelets. In response to this exposure, many recipients can become HLA-sensitized [25, 26].

The mechanism of HLA sensitization from transfusion is complex, depending on the immunologic history of the recipient and might differ from person to person [10]. Sensitization is infrequent and transient when patients receive few blood transfusions [27]. However, one or a few blood transfusions can induce broad and persistent HLA sensitization when given to patients who had undergone clonal expansion due to previous transplants, pregnancies or blood transfusions [28, 29]. Thus, the anti-HLA antibodies in sensitized patient may disappears after some time or remain for long time after blood transfusion [30].

With the introduction of universal leukodepletion of blood components in some countries, such as United Kingdom, France and Canada, there was an assumption that HLA sensitization in renal patients would be minimized. However, generally, universal leucodepletion was not adopted as a routine in many centers in Brazil and most studies have been shown that leukodepletion of blood components reduced the frequency of, but not eliminated, HLA allosensitization [9, 31, 32]. These studies demonstrated that the transfusion with leukodepletion of blood components continues an important risk of HLA sensitization. Because, there are still potential sources of HLA antigens in leuko-depleted blood including HLA molecules present on residual leukocytes, platelets and on superficies of erythrocytes [10, 31, 33] and cross reactivity between red cell antigens [9, 34].

In fact, the HLA sensitization from red blood cell transfusions was often underestimated because to the common misconception that mature erythrocytes do not express any HLA molecules, once they are enucleated cells. It is known that erythrocytes express low levels of HLA class I molecules (100–2,000 per cell), compared to leukocytes (1 -2×10^5) [10, 33, 35], however, because of the large numbers of erythrocytes, HLA sensitization can occur in blood transfusion [35].

Data from this study indicated that most Brazilian candidates continue to receive blood transfusions before transplantation and become sensitized. The high number of patients HLA sensitized can be explained, in part, by the practice for many years, in Brazil, of performing blood transfusions in both hypersensitive and non-sensitized patients without regard to the high probability of sensitization and its possible consequences.

Blood transfusions have been avoided in potential recipients to prevent any possibility of sensitization and the subsequent risk of hyperacute or acute rejection [24]. However there

are circumstances when a blood transfusion may be necessary and mandatory, for example, in acute severe hemorrhage and when there is erythropoiesis-stimulating agents' resistance [10, 11]. However, there are clinical scenarios where they are necessary,

In the current scenario where the majority studies have been demonstrated that leukodepletion of blood products is ineffective in decreasing sensitization [9, 31, 32]; increased HLA sensitization is associated with longer waiting times for finding compatible donors and increased risk of graft failure [2, 24]; desensitization protocols and paired kidney exchanges are limited to only a small proportion of sensitized potential recipients [11], our findings reinforce the importance of weighing the benefits and risks for transfusion in potential transplant candidates, avoiding unnecessary transfusions. Alternatives to avoid blood transfusions should be considered whenever possible [11].

In current study, the prevalence of PRA-positive in patients who not having blood transfusion episode was 35.7% for class I or II, 24.5% for class I, 18.4% for class II and 7.1% for class I and II, most presented PRA lower. This result is higher than that reported in healthy blood donors from United States where the prevalence of anti-HLA antibodies in nontransfused male was 1.0% [36]. Lopes et al., (2015) [37], in Portugal, reported the prevalence of 10% for class I and 5.2% for class II of anti-HLA antibodies in kidney transplantation candidates without any identifiable sensitization event. Discrepant results may occur depending on study, patient population, number and type of transfusions and the use of different methods for detection of anti-HLA antibodies or PRA levels for consideration of HLA sensitization positivity.

Sensitization to HLA antigens in our patients who not having history of a blood transfusion might have arised from an upregulation of the immune system from unconventional source of sensitization such as cross reactivity between artificial surfaces [38], microbial antigens, ingested proteins and allergens [39, 40] and pro-inflammatory events [41]. However, the presence of these anti-HLA antibodies and their clinical relevance need further investigation.

Limitations of this study were the use of small sample sizes and difficulties in obtaining information about previous history of blood transfusions, because not all of the transplant centers and dialysis clinics in Brazil have an informatics system for efficient data storage with updating and controlled retrieval of this information. Despite these limitations, this study revealed important data on the HLA sensitization and transfusion in a population of Brazil.

Conclusions

The data from this study document the sensitization to HLA antigens and transfusion in male Brazilian patients awaiting primary kidney transplant, showing a high proportion of sensitization (positive PRA) and transfusion history in this population, indicating that many candidates continue to receive blood transfusions before transplantation and become sensitized. These findings reinforce the evidence in favor transfusion-minimizing strategies when clinically possible, in potential transplant candidates, and will help clinical decision making with regards to the risk of HLA sensitization after blood transfusion in Brazilian patients awaiting transplantation.

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Disclosure Statement

The authors declare that they have no competing interests.

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Tables

Table 1. Demographic characteristics and transfusion history, according to the PRA results.

	PRA - Positive	**PRA - Negative**	**p value**
n	121	115	
Mean age (years)	50.4 ± 13.8	47.8 ± 13.1	
Ethnic group			
Caucasians	70 (57.9%)	69 (60.0%)	
Mestizos	33 (27.3%)	26 (22.6%)	
Blacks	16 (13.2%)	14 (12.2%)	
Orientals	2 (1.6%)	6 (5.2%)	
Dialysis			
Mean dialysis duration (years)	4.2 ± 3.8	3.4 ± 3.2	
Transfusion			
Any transfusions (nº of patients)	88 (72.7%)	50 (43.5%)	<0.001
Number of transfusion (unit)	3.1 (6.8)	1.8 (3.7)	

PRA, panel-reactive antibody.

Table 2. Anti-HLA class I antibodies in patients with a positive PRA (n = 121).

anti-HLA-A	f	anti-HLA-B	f	anti-HLA-C	f
anti-A1	0.1240	**anti-B7**	0.0714	**anti-C1**	0.0000
anti-A2	0,0744	**anti-B8**	0.0604	**anti-C2**	0.0000
anti-A3	0,0331	**anti-B13**	0.0220	**anti-C4**	0.0083
anti-A11	0.0661	**anti-B18**	0.0549	**anti-C6**	0.0083
anti-A23	0.1074	**anti-B27**	0.0824	**anti-C7**	0.0083
anti-A24	0.1405	**anti-B35**	0.0549	**anti-C8**	0.0083
anti-A25	0.0744	**anti-B37**	0.0110	**anti-C9**	0.0000
anti-A26	0.0496	**anti-B38**	0.0330	**anti-C10**	0.0000
anti-A29	0.0579	**anti-B39**	0.0824	**anti-C12**	0.0165
anti-A30	0.0579	**anti-B41**	0.0220	**anti-C14**	0.0000
anti-A31	0.1074	**anti-B42**	0.0385	**anti-C15**	0.0248
anti-A32	0.0909	**anti-B44**	0.0604	**anti-C16**	0.0331
anti-A33	0.0744	**anti-B45**	0.0714	**anti-C18**	0.0083
anti-A34	0.1901	**anti-B46**	0.0055		
anti-A36	0.0661	**anti-B47**	0.0055		
anti-A66	0.1322	**anti-B48**	0.0659		
anti-A68	0.1074	**ant-B49**	0.0549		
anti-A69	0.0661	**anti-B50**	0.0440		
anti-A74	0,0248	**anti-B51**	0.0275		
anti-A80	0.0413	**anti-B52**	0.0714		
		anti-B53	0.0275		
		anti-B54	0.0440		
		anti-B55	0.0604		
		anti-B56	0,0824		
		anti-B57	0.0495		
		anti-B58	0.0385		
		anti-B59	0.0110		
		anti-B60	0.0769		
		anti-B61	0.0659		
		anti-B62	0.0275		
		anti-B63	0.0495		

anti-B64	0.0385
anti-B65	0.0549
anti-B67	0.0604
anti-B71	0.0275
anti-B72	0.0220
anti-B73	0.0055
anti-B75	0.0165
anti-B76	0.0330
anti-B78	0.0275
anti-B81	0.0604
anti-B82	0.0330

PRA, panel-reactive antibody; f, frequency.

Table 3. Anti-HLA class II antibodies in patients with a positive PRA (n = 121).

anti-HLA-DR	f	anti-HLA-DQ	f	anti-HLA-DP	f
anti-DR1	0.1157	**anti-DQ2**	0.0331	**anti-DP1**	0.0000
anti-DR4	0.1322	**anti-DQ4**	0.0496	**anti-DP2**	0.0000
anti-DR7	0.1405	**anti-DQ5**	0.0331	**anti-DP3**	0.0083
anti-DR8	0.2066	**anti-DQ6**	0.0579	**anti-DP4**	0.0000
anti-DR9	0.1736	**anti-DQ7**	0.1653	**anti-DP5**	0.0000
anti-DR10	0.0744	**anti-DQ8**	0.2479	**anti-DP13**	0.0083
anti-DR11	0.0661	**anti-DQ9**	0.2231	**anti-DP14**	0.0000
anti-DR12	0.2397			**anti-DP18**	0.0083
anti-DR13	0.0909				
anti-DR14	0.0744				
anti-DR15	0.1240				
anti-DR16	0.1818				
anti-DR17	0.0992				
anti-DR18	0.0744				
anti-DR51	0.1405				
anti-DR52	0.0413				
anti-DR53	0.0826				
anti-DR103	0.1074				

PRA, panel-reactive antibody; f, frequency.

Table 4. Demographic characteristics and PRA results, according to the transfusion history.

	Transfused	**Nontransfused**	**p value**
n	138 (58.5%)	98 (41.5%)	
Mean age (years)	48.7 ± 13.6	49.7 ± 13.3	
Ethnic group			
Caucasians	75 (54.4%)	64 (65.3%)	
Mestizos	37 (26.8%)	22 (22.5%)	
Blacks	21 (15.2%)	9 (9.2%)	
Orientals	5 (3.6%)	3 (3.0%)	
Dialysis			
Mean dialysis duration (years)	4.4 ± 3.8	3.0 ± 3.0	<0.01
PRA CI or CII			<0.001
Positive	88 (63.8%)	35 (35.7%)	
Negative	50 (36.2%)	63 (64.3%)	
PRA CI			
Positive	64 (46.4%)	24 (24.5%)	<0.001
1-50%	49 (76.6%)	22 (91.7%)	
51-100%	15 (23.4%)	2 (8.3%)	
PRA CII			
Positive	50 (36.2%)	18 (18.4%)	<0.01
1-50%	36 (72.0%)	17 (94.4%)	
51-100%	14 (28.0%)	1 (5.6%)	
PRA CI and CII positive	26 (18.8%)	7 (7.1%)	0.013
Anti-HLA antibodies			
median number of HLA Class I specific antibody	3.2 ± 6.8	1.2 ± 3.5	<0.01
median number of HLA Class II specific antibody	2.1 ± 3.8	0.7 ± 1.9	<0.001
median number of HLA Class I and Class II specific antibody	5.4 ± 9.0	1.8 ± 4.2	<0.001

PRA, panel-reactive antibody.

2. CAPÍTULO III

3.1. CONCLUSÕES

Como opção, apresentamos neste tópico as conclusões gerais da tese. As conclusões específicas estão ao longo dos três artigos que, compõem este trabalho.

Este estudo sobre genes HLA e resposta imune humoral aos antígenos HLA mostrou que:

- O desempenho do método de citotoxicidade dependente de complemento com a adição de antiglobulina humana (CDC-AGH) para a detecção de anticorpos anti-HLA foi comparável ao Imunoensaio de fase sólida utilizando tecnologia Luminex (IFS) na avaliação de PRA classe I.
- Alguns grupos alélicos HLA de classe I e de classe II podem estar envolvidos na diminuição da resposta imune humoral aos antígenos HLA.
- Muitos pacientes continuam a receber transfusões sanguíneas antes do transplante, aumentando a possibilidade de se tornarem sensibilizados.

Os dados deste estudo serviram como base para o conhecimento do polimorfismo dos genes HLA e da resposta imune humoral aos antígenos HLA em candidatos ao transplante renal da região Norte/Noroeste do Estado do Paraná, Brasil.

3.2. **PERSPECTIVAS FUTURAS**

A identificação de grupos alélicos HLA envolvidos na sensibilização HLA poderá contribuir para o entendimento de futuros eventos relacionados à aceitação ou rejeição do enxerto. A avaliação da resposta imune aos antígenos HLA na fase pré-transplante poderá auxiliar a equipe transplantadora no cuidado ao pacientes renal crônico referente às imunizações, naturais e artificiais, bem como na terapia supressora a ser adotada caso o paciente renal crônico seja transplantado.