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COMPARAÇÃO ENTRE OS MÉTODOS DE ANTIGENEMIA E DE PCR EM TEMPO REAL PARA CITOMEGALOVÍRUS EM TRANSPLANTADOS RENAIS

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Dissertação submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre

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Resumo da Dissertação

Introdução: O citomegalovírus (CMV) é considerado o patógeno de maior importância clínica em pacientes transplantados renais. **Objetivos:** Desenvolver e validar um ensaio *in-house* de PCR em tempo real (qPCR) para CMV calibrado com o 1º Padrão Internacional da Organização Mundial da Saúde e comparar com o exame de antigenemia em uma coorte de pacientes transplantados renais. Além disso, estabelecer ponto de corte para início da terapia preemptiva. **Material e Métodos:** Para desenvolvimento e validação do ensaio foram realizados testes de otimização, sensibilidade e especificidade analíticas, precisão e reprodutibilidade interlaboratorial. A comparação entre as metodologias foi feita por análise descritiva, coeficiente de Kappa e correlação de Spearman. Foi realizada uma análise de curva ROC para determinar um ponto de corte para início de tratamento. **Resultados:** O ensaio desenvolvido se mostrou sensível, específico, preciso e linear. 232 amostras de 30 pacientes foram analisadas para antigenemia e qPCR. Os resultados mostraram que 163 (70,26%) amostras eram concordantes. Dos 69 com resultados discordantes, 54 (78,26%) foram positivos no teste de qPCR dias antes ou depois da antigenemia. O teste qPCR apresentou mediana de 12 dias (0–25) para positivação antes da antigenemia e de 9 dias (0–28) para negativação após a antigenemia. O coeficiente Kappa apresentou concordância justa (0,435; p <0,001), a correlação de Spearman se apresentou moderada (0,663; p <0,001). A análise da curva ROC encontrou um ponto de corte de 3.430 UI/ml (Log 3,53) (p <0,001) com base em 10 células/10^5 leucócitos na antigenemia e na decisão de tratamento pelo médico. **Conclusão:** As metodologias apresentaram concordância justa e correlação moderada. O qPCR apresentou-se como um

teste sensível, permitindo melhor avaliação da DNAemia nos pacientes, obtendo resultados positivos semanas antes da antigenemia. O ponto de corte estabelecido neste estudo permite a validação do teste para a população de nossa instituição, como sugerem diretrizes internacionais.

Palavras-chave: Citomegalovírus, infecção por CMV, doença por CMV, PCR quantitativo em tempo real, antigenemia.

Abstract

Introduction: Cytomegalovirus (CMV) is considered the most clinically important pathogen in kidney transplant patients. **Objectives:** To develop and validate a real-time in-house PCR (qPCR) assay for CMV calibrated with the 1st WHO International Standard for Human Cytomegalovirus and compare with the antigenemia test in a cohort of kidney transplant patients. Also, establish a threshold for initiation of preemptive therapy. **Material and Methods:** For development and validation of the assay the following tests were performed: optimization, analytical sensitivity and specificity, precision and interlaboratory reproducibility. Methodologies were compared by descriptive analysis, Kappa coefficient and Spearman correlation. A ROC curve analysis was performed to determine a threshold for treatment initiation. **Results:** The in-house assay was sensitive, specific, precise and linear. 232 samples from 30 patients were analyzed for antigenemia and qPCR. Results showed that 163 (70.26%) samples were concordant. Of 69 samples with discordant results, 54 (78.26%) were positive on the qPCR assay days before or after antigenemia. The qPCR test presented a median of 12 days (0–25) for positivity before antigenemia and 9 days (0–28) for negativity after antigenemia. The Kappa coefficient showed fair agreement (0.435; p <0.001), Spearman correlation was moderate (0.663; p <0.001). ROC curve analysis found a threshold of 3,430 IU / ml (Log 3.53) (p <0.001) based on 10 cells/10^5 leukocytes in antigenemia and the physician's decision to treat. **Conclusion:** The methodologies showed fair agreement and moderate correlation. The qPCR was presented as a sensitive test, allowing a better evaluation of the DNAemia in the patients, obtaining positive results weeks before the antigenemia. The cutoff point established in this study allows

the validation of the test for the population of our institution, as suggested by international guidelines.

Keywords: Cytomegalovirus, CMV infection, CMV disease, quantitative real time PCR, antigenemia.

Lista de abreviaturas

CMV: Citomegalovírus

PCR: (do inglês: Polymerase Chain Reaction) Reação em Cadeira da Polimerase

qPCR: Reação em Cadeia da Polimerase Quantitativa em Tempo Real

CD34+: Cluster of Differentiation 34

CD14+: Cluster of Differentiation 14

ISCMPA: Irmandade Santa Casa de Misericórdia de Porto Alegre

UFCSPA: Universidade Federal de Ciências de Saúde de Porto Alegre

D+/ R-: Doador Soropositivo para CMV e Receptor Soronegativo para CMV

D+/ R+: Doador Soropositivo para CMV e Receptor Soropositivo para CMV

NAT: Técnica de Amplificação de Ácido Nucléico

DNA: Ácido Desoxirribonucléico

UI/mL: Unidades Internacionais por mL

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REFERENCIAL TEÓRICO

1.1 Transplante renal

Os rins têm como principais funções filtrar o sangue, regular a pressão arterial, produzir hormônios, participar na formação e na manutenção óssea e estimular a produção de eritrócitos. Patologias como diabetes, hipertensão arterial, cálculos renais, nefrites, malformações do sistema urinário e infecções urinárias de repetição podem afetar suas funções (1–5).

Os danos causados aos rins podem ser tratados, em casos menos graves, por meio de medicamentos e, nos casos extremos, quando é necessária a substituição da função renal, por duas opções: a diálise e o transplante. O transplante renal se apresenta como a melhor opção terapêutica, tendo em vista o custo-benefício, a sobrevida e a qualidade de vida dos pacientes (1–5).

O Brasil é o segundo país com o maior número absoluto de transplantes renais no mundo, totalizando o equivalente a 5.929 operações em 2017. De janeiro de 2008 a dezembro de 2018 foram realizados, em nosso país, cerca de 57.275 transplantes de rim. Contudo, encontravam-se na lista de espera, em dezembro de 2018, 22.581 pacientes, o que evidencia a importância dessa metodologia (6). A Irmandade Santa Casa de Misericórdia de Porto Alegre (ISCMPA), é referência em transplantes no Brasil e na América Latina. Em novembro de 2015, foram comemorados quatro mil transplantes renais realizados, desde 1977, quando o programa de transplantes de rim da instituição foi implementado (7).

1.2 Citomegalovírus no Transplante Renal

O citomegalovírus (CMV) é um vírus que possui genoma de ácido desoxirribonucleico (DNA), e pertence à subfamília *β-herpes* da família *Herpesviridae*, sendo o maior herpes-vírus humano conhecido (8). A primo-infecção por este patógeno geralmente ocorre na infância, sua prevalência mundial vai de 30–100% (9–14). Após a primeira infecção o vírus permanece latente em subpopulações de progenitores mielóides CD34+, monócitos CD14+, células dendríticas e megacariócitos(9,15).

O CMV é de grande importância clínica para pacientes transplantados, em especial os pacientes transplantados renais, sendo responsável por altas taxas de morbidade e mortalidade (16,17). A infecção pelo vírus pode ocorrer devido a reativação do CMV latente, a transmissão pelo órgão transplantado e, também, a reinfecção por diferentes sorotipos (18).

Esse patógeno pode causar efeitos diretos e indiretos. Os efeitos diretos são a infecção e a doença, sendo a infecção a replicação do vírus sem a sintomatologia e doença a replicação associada à apresentação de sintomas(19). A doença por CMV é caracterizada por sintomas de síndrome viral (febre, mal-estar, leucopenia e/ou trombocitopenia) ou por doença invasiva tecidual (19). Já os efeitos indiretos são nefropatia crônica do enxerto e/ou perda do enxerto, infecções bacterianas, infecções fúngicas, infecções virais, desordem linfoproliferativa pós-transplante, diabetes mellitus de início recente após o transplante, imunossenescência e rejeição aguda (20–35).

Os fatores de risco para infecção e doença por CMV estão relacionados ao soro *status* do doador e do receptor e a fatores do hospedeiro. O soro *status* considerado de alto risco é representado pelo doador soropositivo para CMV e

o receptor soronegativo (D+/ R-), tornando altas as chances da primeira infecção. Os fatores relacionados ao hospedeiro são a idade, a presença de comorbidades, leucopenia, linfopenia, fatores genéticos e grau de imunossupressão (36,37).

1.3 Diagnóstico do CMV no Transplante Renal

O monitoramento para CMV começa no pré-transplante, em que são realizados testes sorológicos a fim de classificar o receptor e o doador, uma vez que o soro *status* é considerado um marcador de prognóstico, tendo em vista que D+/ R- possuem maior risco de perda do enxerto, de morrer por todas as causas ou morrer por infecção viral (38). Já no pós-transplante, o enfoque diagnóstico é voltado para infecção e para doença. As principais técnicas utilizadas são: histopatológica, cultura, antigenemia e ensaios moleculares para detecção e quantificação de ácido nucléico para CMV (NAT) (39–44).

A técnica de histopatologia é utilizada parta confirmar a doença invasiva por CMV, todavia esta metodologia vem sendo substituída por métodos menos agressivos, sendo recomenda para o diagnóstico de doença invasiva (45,46). Quanto à cultura viral, apesar de sua alta especificidade, possui uma baixa utilidade clínica, tendo em vista sua baixa sensibilidade (41).

As técnicas de antigenemia e de reação em cadeia da polimerase (PCR), são fundamentais para o diagnóstico e monitoramento de infecção e da doença por CMV (47). Essas metodologias podem ser utilizadas para o acompanhamento das terapias profiláticas e preemptivas, bem como para cessá-las (48). Atualmente, a PCR quantitativa em tempo real (qPCR) para CMV é o padrão ouro para o diagnóstico, entretanto laboratórios com recursos econômicos limitados ainda utilizam a antigenemia (49).

1.4 Antigenemia para CMV

A antigenemia é um teste de imunofluorescência semiquantitativo, que consiste na detecção do antígeno Pp65 do CMV nos leucócitos no sangue periférico (40,42,50,51). Três tipos de antígeno são produzidos pelo vírus na fase de replicação: os imediatos, 1 a 3 horas após a infecção e que permanecem durante a fase latente; os precoces, 3 horas após a infecção; os antígenos tardios, que aparecem após a síntese do DNA, representando assim a infecção ativa. O Pp65 é um antígeno tardio que é captado no citoplasma de leucócitos (8).

Essa técnica tornou possível o monitoramento do CMV nos pacientes após o transplante e foi largamente utilizada até o desenvolvimento dos NATs. Diversas vantagens podem ser apresentadas como: rapidez dos resultados, alta sensibilidade quando comparada à cultura viral, indica infecção ativa antes do aparecimento dos sintomas, não necessita de equipamentos caros e o ensaio é relativamente fácil de ser realizado (39,46,51–54). Conquanto, desvantagens são apontadas, tais como a falta de padronização da técnica, a subjetividade na interpretação dos resultados e a necessidade do processamento do sangue dentro de 6–8 horas após a coleta. Além disso, o ensaio não pode ser realizado em pacientes neutropênicos com contagem menor que 1000/mm^3 (46,52,53).

Estudos vêm sendo realizados na ISCMPA desde 1993 visando estabelecer os pontos de cortes mais adequados para utilização da antigenemia para tratamento dos pacientes no pós-transplante. A implementação desse método reduziu a incidência da doença por CMV na instituição (39). Contudo, o panorama mundial indica que grande parte dos

laboratórios já migraram para o NAT, tendo em vista sua sensibilidade e especificidade superior. A antigenemia permanece sendo utilizada em grande escala na Ásia e em laboratórios com menos recursos financeiros (48).

1.5 PCR em Tempo Real Para CMV

A qPCR para CMV se tornou o teste padrão ouro para o diagnóstico de infecção e de doença, podendo utilizar sangue total ou plasma como matriz biológica (47,55). Essa metodologia detecta e quantifica ácidos nucleicos do CMV, sendo que a presença de DNA do vírus geralmente é indicativo de replicação viral (37,56). É importante ressaltar que a presença de ácidos nucleicos de CMV não expressa, necessariamente, infecção ativa, o que demonstra a necessidade de estabelecimento de pontos de corte corretos para o tratamento (57).

Essa metodologia pode ser utilizada para decisão terapêutica, monitoramento da terapia preemptiva e verificação de resistência a medicamentos. A versatilidade desse teste permite, ainda, a validação de ensaios *multiplex* com outros patógenos de interesse (47,58–60).

As vantagens apresentadas por esse método são melhor precisão, maior intervalo linear, maior sensibilidade, menor complexidade, maior número de reagentes disponíveis, menor tempo para realização do ensaio, possibilidade de estocagem da amostra, maior reprodutibilidade e mais fácil interpretação (47,52,61,62).

As desvantagens dessa metodologia são a necessidade de equipamentos e reagentes caros, a pouca padronização analítica e a variação dos ensaios (63). A padronização desta metodologia ainda representa um desafio à comunidade internacional, mesmo após o lançamento do padrão da

OMS. Para modificar esta realidade, mais estudos são necessários visando estabelecer pontos de cortes em diferentes populações, bem como a validação de diferentes ensaios de qPCR que permitirão a avaliação de fatores que interferem no desenho dos testes.

1.6 Tratamento e Prevenção

A prevenção para o CMV consiste em duas estratégias: a profilaxia universal e a terapia preemptiva (19). A profilaxia universal resume-se na administração de antiviral para todos os pacientes ou para grupos considerados de risco, de três a seis meses após o transplante (64). Essa metodologia apresenta vantagens e desvantagens; entre as vantagens se destaca a fácil administração dos medicamentos, a proteção contra outros herpesvírus e a diminuição da incidência dos fatores indiretos causados pelo CMV; as desvantagens apresentadas são a toxicidade relacionada ao medicamento e a doença de início tardio pelo CMV (58,65,66). A terapia preemptiva ocorre por meio do monitoramento laboratorial (antigenemia ou qPCR) em intervalos regulares, visando detectar a replicação viral antes do aparecimento dos sintomas. Assim que o aumento da replicação atingir um certo ponto de corte, inicia-se o tratamento para evitar a progressão para doença (63). As vantagens dessa abordagem são a diminuição da toxicidade associada aos medicamentos e seus custos, menor risco de desenvolvimento de doença tardia e diminuição da ocorrência de resistência. Quanto às desvantagens, observa-se o aumento dos custos laboratoriais, a dificuldade na frequência de consultas e avaliações laboratoriais e a intervenção em tempo hábil de impedir a progressão para a doença (30,67–72).

1.7 Justificativa

O CMV é um vírus de grande importância clínica para pacientes transplantados renais, tendo em vista possível transmissão desse patógeno pelo órgão transplantado ou pela reativação do vírus em função da imunossupressão. A prevalência mundial do CMV varia de 30–100%, como apresentado anteriormente, e duas principais metodologias são aceitas para monitoramento e diagnóstico do CMV no pós-transplante renal: a antigenemia e os NATs. Antes do desenvolvimento das metodologias moleculares, a antigenemia era a estratégia mais utilizada, hoje a qPCR é considerada o padrão ouro para o diagnóstico de CMV. Todavia, em função dos altos custos dos testes comerciais de biologia molecular e da limitação orçamentária, alguns laboratórios ainda optam pela utilização da antigenemia. Outro ponto a ser enfatizado é a difícil padronização analítica e clínica das metodologias moleculares para este patógeno, mesmo após o lançamento do padrão internacional da OMS. Portanto, visando melhorar o monitoramento e diagnóstico do CMV na população de pacientes transplantados renais da ISCMPA, bem como desenvolver uma metodologia com custo acessível a necessidade da instituição, esse estudo apresenta a validação de uma qPCR e de um ponto de corte para início do tratamento em uma coorte de pacientes transplantados renais.

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3. OBJETIVOS

O objetivo geral deste estudo foi:

a) Comparar as metodologias de antigenemia e de PCR em tempo real no pós-transplante renal para a detecção de CMV.

Além disso, os objetivos específicos para esta pesquisa foram:

b) Determinar pontos de corte para o método de PCR em tempo real para CMV, em amostras de plasma, que possam predizer o início do tratamento;
c) determinar o custo para ambos os testes;
d) determinar a precisão da metodologia de qPCR *in-house*;
e) determinar a linearidade da metodologia de qPCR *in-house*;
f) determinar a sensibilidade analítica da metodologia de qPCR *in-house*;
g) determinar a especificidade analítica da metodologia de qPCR *in-house*;
h) determinar a sensibilidade diagnóstica da metodologia de qPCR *in-house*;
i) determinar a especificidade diagnóstica da metodologia de qPCR *in-house*;
j) avaliar os fatores de risco para CMV nos transplantados renais na Santa Casa;
k) avaliar a incidência de infecção ativa por CMV nos primeiros três meses pós-transplante renal, por qualquer dos métodos diagnósticos empregados.

4. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

4.1 ARTIGO 1

"Cost minimization analysis and validation of a quantitative in-house real time PCR assay in relation to an automatized method for cytomegalovirus"

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Cost minimization analysis and validation of a cytomegalovirus quantitative in-house real time PCR assay in relation to m2000 RealTime System

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ABSTRACT

Introduction: Cytomegalovirus (CMV) causes severe diseases in the immunocompromised patient. Nowadays, quantitative polymerase chain reaction (qPCR) is the gold-standard for diagnosis and monitoring of CMV infection. **Objective:** This study aims to validate an in-house CMV qPCR assay calibrated against the World Health Organization Standard and to perform a cost minimization analysis for the perspective of the laboratory. **Study design:** The methodology consisted of determining: optimization, analytical sensitivity and specificity, precision and inter laboratorial reproducibility. Patients (n=30) with known results for CMV tested with m2000 RealTime System (Abbott Laboratories, USA) were tested with the in-house assay, as well as patients with known infection for other human herpes and BK virus. A cost minimization analysis was performed due to the diagnostic equivalence of the methodologies applied in the study. **Results:** The in-house assay had a limit of detection and quantification of 60.26 IU/mL, with no cross-reactivity with other agents. Besides that, the test was precise and had a R^2 of 0.954 when compared with m2000 equipment. The cost analysis shows that the assay was most economically advantageous with median of 45.8% (range 45.5–49.2%) and 77.8% (range 76.7–85.0%) than the test used in the hospital and the m2000 equipment respectively. **Conclusions:** These results showed that in-house qPCR testing is a possible alternative in comparison to automated platforms, being considerably less expensive and as efficacious as the commercial methods.

KEYWORDS: Cytomegalovirus; cost; in-house; PCR; pharmacoeconomics; standardization.

INTRODUCTION

Cytomegalovirus (CMV) is the largest Human Herpesvirus, which has been associated with severe infections in immunocompromised patients. The main populations at risk are HIV patients (1), pregnant women (vertical transmission) (2) and individuals who have undergone hematopoietic (3) or solid organs transplants (4).

In the 1990s, the methodology of choice for diagnosing and monitoring CMV infection/disease was CMV Pp65 antigenemia (5–8). However, due to known limitations of this technique and the advent of molecular diagnosis, polymerase chain reaction (PCR) for CMV became the test of choice, with the possibilities of qualitative and quantitative results (9,10). Molecular techniques have revolutionized the diagnosis of infectious diseases, improving the diagnostic speed and accuracy in comparison to other methodologies already in place (11). Nevertheless, several publications demonstrated low reproducibility and standardization of the technique (12,13). In order to improve the performance of CMV PCR tests, in 2010 the World Health Organization (WHO) provided the 1st WHO International Standard for Human Cytomegalovirus (WHO IS) (14). Almost 10 years after the release of this standard, there are few publications that evaluated the standardization of in-house tests for the diagnosis of CMV infection, using the WHO IS as a standard (15–20). The validation of in-house molecular tests may allow laboratories in developing countries to offer services at a reduced cost in comparison to commercial or automated platforms (17). This article follows the recommendations of the MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines and is the first study that compares different molecular methodologies for cytomegalovirus detection with an economic analysis.

Therefore, this study aims to present a validation of a quantitative in-house real time PCR for CMV comparing with an automated platform using a cost minimization analysis.

MATERIALS AND METHODS

Patients and samples

Samples were those originated from patients for whom molecular tests were requested at Hospital de Clínicas de Porto Alegre (HCPA) and Santa Casa de Misericórdia de Porto Alegre (ISCMPA), both large tertiary hospitals located in Porto Alegre, Southern Brazil. All samples used for the study were collected in 4 mL EDTA tubes with separator gels, centrifuged at 1300 xg for 15 min and frozen at -80ºC until nucleic acid extraction.

Nucleic acid extraction

Plasma samples were extracted with Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, USA) following the manufacturer's instructions. The volume of plasma used for extraction was 300 µL and the elution volume was 50 µL.

Quantitative in-house real time PCR optimization

Probes and primers sequences are shown in the supplementary material (21). Both primers and probes were synthesized by Síntese Biotecnologia (Thermo Scientific, BR) with amplicon sizes of 105 bp (UL 34) and 98 bp (UL 80.5).

Real time PCR (qPCR) tests were first optimized in simplex reactions, in order to determine the optimum primer and probe concentrations, as well as the ideal amount of DNA and reagents to be used in the assay. Primers were

evaluated in the range of 0.3–0.5 µM, and probes were tested in concentrations ranging from 0.20–0.30 µM. Different amounts of eluted DNA were tested, from 2–4 µL of extracted material. Different PCR master mixes were also evaluated: GoTaq Probe qPCR Master Mix (Promega, BR), Platinum® Quantitative PCR SuperMix-UDG (Thermo Scientific, BR) and TaqMan® Gene Expression Master Mix (Thermo Scientific, BR). In addition, different concentrations of Reference Dye were tested, from 1:10 to 1:500. The thermocycling conditions for the qPCR reactions were: 1 cycle of 2 minutes at 50 °C; 2 min at 95 °C; followed by 40 cycles of 15 sec at 95 °C, and 1 min at 60 °C, in a 7500 real time PCR system (Thermo Scientific, BR).

The standard curve used for secondary calibration standard was inserted into a plasmid, synthesized by Applied Biosystems (Thermo Scientific, BR). Sequences are shown in the supplementary material.

Calibration curves

The primary calibration standard used in this study was the 1st WHO International Standard for Human Cytomegalovirus NCBI code 09/132. Material was eluted in 1 mL of nuclease-free water as indicated by the manufacturer, containing an initial concentration of 5 x 10^6 IU/mL. From this material, two aliquots were prepared for extraction, one containing 500 µL of the standard diluted 1:2 in buffer (TE) and another in a 1:2 dilution of the CMV-free plasma in a final volume of 500 µL to mimic the biological matrix.

The secondary pattern used in the study was a plasmid designed for the target both primers and probes sequences. The standard had an initial concentration of 9.65 x 10^{10} copies/mL.

Analytical sensitivity

To determine the limit of quantification (LOQ), two different operators performed the analytical sensitivity test on three distinct days. The test consisted in a *simplicata* curve which was amplified in parallel for a base 10 dilution of the primary standard diluted in buffer (TE) (initial concentration of 2.5 x 10^6 IU/mL), the primary standard diluted in negative CMV plasma (initial concentration 2.5 x 10^6 IU/mL) and the secondary standard (initial concentration of 9.65 x 10^5 copies/mL). The standard dilution test and DNA extraction were also performed on three distinguished days.

For the determination of the limit of detection (LOD), the lower point of the curve amplified by 95% of the time was diluted in base two, in triplicate. The concentration that consistently amplified 95% of the time was tested again, in triplicate.

The conversion factor was calculated by dividing the IU/mL amount from the primary standard, taking into account an estimated 80% efficiency in extraction, by the average number of copies/mL, for both genes, found in the three days of the test for each of the points of the curve of the secondary pattern. The median of these results was calculated.

Analytical specificity

For the determination of the analytical specificity, plasma samples from patients of ISCMPA infected with herpes simplex (HSV)-1, HSV-2, Human Herpesvirus (HHV)-3, HHV-4, HSV-6, HSV-7 and BK virus (BKV) were extracted and tested.

Precision

The precision test was performed with two patient’s samples from HCPA with low (138 IU/mL) and high concentration (20,508 IU/mL) of CMV DNA,

according to m2000 (Abbott Laboratories, USA) quantification in triplicate, in five different days. The cycle threshold (Ct) for each run was determined and the precision was analyzed by calculating the standard deviation (SD) and coefficient of variation (CV) for the replicates in the same day and in different days, in order to evaluate the intra and inter assay variability.

Curve variability analysis

Ct variability in the secondary pattern curve analyses were performed along ten different days, in simple reactions. The curve consists of 5 points, ranging from 9.65×10^5 to 96.5 copies/mL.

Interlaboratorial reproducibility

This experiment was conducted with 30 samples from HCPA that had been tested for CMV viral load, using the m2000 equipment (Abbott Laboratories, USA). For these samples, 10 had an undetected result when tested with the m2000; 10 had low concentrations, ranging from 47 IU/mL (1.67 log IU/mL) to 490 IU/mL (2.69 log IU/mL); and 10 were at high concentrations, ranging from 976 IU/mL (2.99 log IU/mL) to 20,508 IU/mL (4.31 log IU/mL). The log difference was calculated to evaluate the performance of the test.

Assay costs

All results are expressed in US dollars, USD, considering mean USD conversion rate for the studied period (December 1st, 2016 to December 31st, 2017) 1 USD to 3.2 BRL (Brazilian Real) (22). To assess the cost of each test, direct and indirect costs for testing were taken into account. Direct costs were those related to the work time of the professionals, the materials used to perform the test, other materials used by the laboratory, equipment depreciation and overall laboratory costs. Indirect costs were related to third-party services

(proficiency assay costs). Costs were calculated for the in-house assay, m2000 RealTime System (Abbott Laboratories, BR) assay, as well as for the test currently used in the hospital: Kit XGEN Master CMV (Mobius Life Science Product Trade for Laboratories Ltda, BR). A cost minimization analysis and a sensitivity test were performed, from a perspective of the laboratory, to evaluate the reduction of costs in the utilization of each alternative.

Ethics

The present study was approved by ethic committees in accordance with the precepts of the Declaration of Helsinki by the following protocol numbers: 1.820.875 and 1.885.683.

RESULTS

Optimization

The qPCR optimization tests showed that the optimal concentration of reagents were: primers 0.4 µM, probes 0.25 µM, and DNA 3 µL, respectively. All reagents tested were adequate for amplification of the secondary pattern, and no amplification was seen with the primary standard for Platinum® Quantitative PCR SuperMix-UDG reagent (Applied Biosystems, USA). Therefore, taking into consideration the performance (early Ct) and cost of the reagents, GoTaq Probe qPCR Master Mix (Promega, USA) was chosen as the master mix for the assay. The results of the Cts for the negative control were undetected for all reagents, for the primary standard the Cts were undetected for the Platinum® Quantitative PCR SuperMix-UDG reagent, 24.35 (UL34) and 23.19 (UL80.5) for GoTaq Probe qPCR Master Mix and 27.87 (UL34) and 26.17 (UL80.5) for TaqMan® Gene Expression Master Mix. The secondary standard showed Cts results of 13.79 (UL34) and 13.74 (UL80.5) for Platinum®

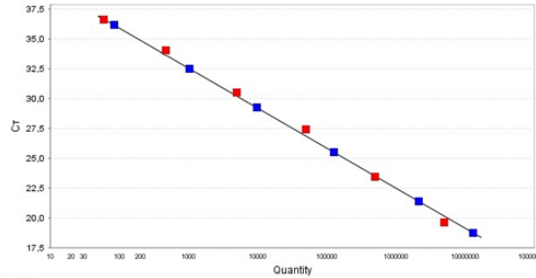
Quantitative PCR SuperMix-UDG reagent, 14.86 (UL34) and 14.42 (UL80.5) for GoTaq Probe qPCR Master Mix and 17.35 (UL34) and 16.37 (UL80.5) for TaqMan® Gene Expression Master Mix. Finally, the reference dye carboxy-X-rhodamine (CXR) tests showed that the ideal dilution was 1:50.

Analytical sensitivity

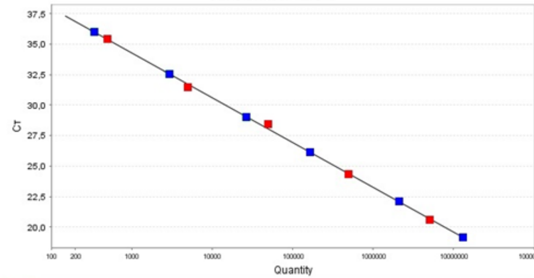
The tests performed with the primary standard diluted in the biological matrix revealed no amplification in the last 3 points of the curve. The curves performed did not show $r^2 > 0.99$ and adequate slope or efficiency. The r^2 results were 0.931, 0.934, 0.933, 0.933, 0.904, and 0.998, with slope of 3.694, 3.224, 3.726, 3.211, 2.62 and 3.474 and efficiency of 86.507, 104.332, 85.526, 104.861, 140.788, and 94.023. Therefore, calibrations for the secondary standard were performed with the primary standard diluted in buffer, and the results of the qPCR parameters are shown in the Figure 1. The analytical sensitivity for the determination of the threshold for each of the genes was 0.21 (UL34) and 0.12 (UL80.5). The conversion factor was 0.29. The LOQ and the LOD of the test was the same, 60.26 IU/mL.

UL 34

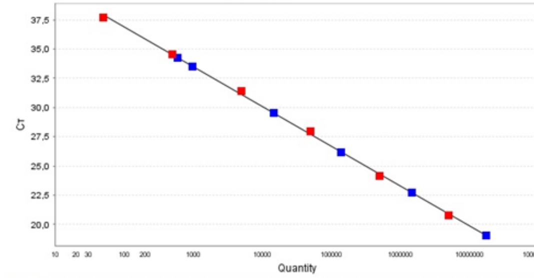
A) Slope: *-3.35* **Y-inter:** *42.63* **R²:** *0.99* **Eff:** *98.83*



B) Slope: *-3.39* **Y-inter:** *43.81* **R²:** *0.99* **Eff:** *97.21*

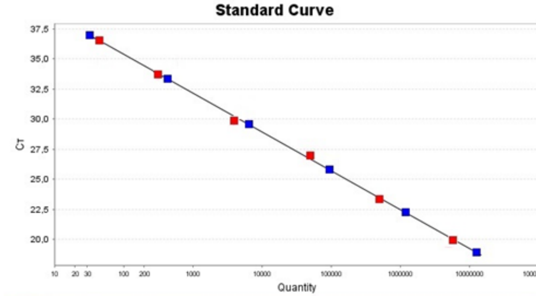


C) Slope: *-3.41* **Y-inter:** *43.73* **R²:** *1* **Eff:** *96.63*

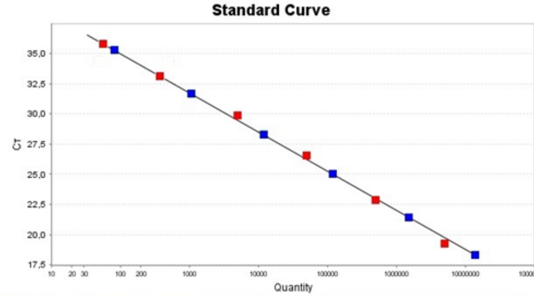


UL 80.5

D) Slope: *-3.55* **Y-inter:** *42.74* **R²:** *0.99* **Eff:** *91.43*



E) Slope: *-3.38* **Y-inter:** *43.89* **R²:** *0.99* **Eff:** *97.82*



F) Slope: *-3.29* **Y-inter:** *41.83* **R²:** *0.99* **Eff:** *101.19*

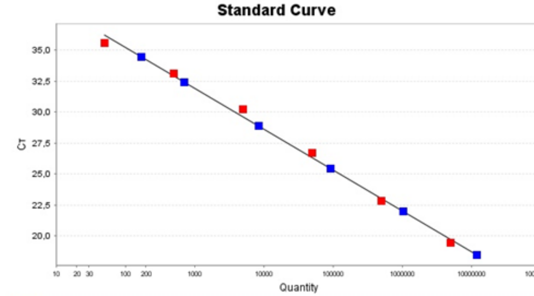


Figure 1. Analytical sensitivity assays for the two CMV genes, UL34 and UL 80.5, performed in three consecutive days.

Analytical specificity

No cross-reactivity was observed in the analytical specificity assay for the other viruses tested in this study.

Precision

For the precision test evaluation an intra- and inter-assay evaluation of the Ct results of both CMV genes for each of the samples: low (138 IU/mL) and high concentrations (20,508 IU/mL) of CMV DNA was performed, as shown in Tables 1, 2, 3 and 4.

Table 1. Precision test for the cytomegalovirus UL34 gene in low concentration sample. Numbers represent cycle threshold (Ct) values.

	Intra Assay					**Inter Assay**		
Parameters	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV%**
	35.70	35.27	35.18	36.24	34.88	35.27	0.74	2.11
	35.22	35.00	34.89	36.01	34.99			
	36.54	36.05	34.88	34.63	33.59			
Mean	35.82	35.44	34.98	35.63	34.49			
SD[a]	0.67	0.55	0.17	0.87	0.78			
CV%[b]	1.87	1.54	0.49	2.44	2.26			

[a]: Standard deviation
[b]: Coefficient of variation

Table 2. Precision test for the cytomegalovirus UL80.5 gene in low concentration sample. Numbers represent cycle threshold (Ct) values.

	Intra Assay					**Inter Assay**		
Parameters	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV%**
	34.87	33.78	35.53	34.04	34.32	34.68	1.01	2.90
	36.21	35.62	34.94	33.30	34.79			
	34.03	34.18	35.92	35.84	32.87			
Mean	35.04	34.53	35.46	34.39	33.99			
SD	1.10	0.97	0.49	1.31	1.00			
CV%	3.14	2.80	1.39	3.80	2.94			

[a]: Standard deviation
[b]: Coefficient of variation

Table 3. Precision test for the cytomegalovirus UL34 gene in high concentration sample. Numbers represent cycle threshold (Ct) values.

	Intra Assay					**Inter Assay**		
Parameters	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV%**
	29.45	29.33	29.81	29.40	29.57	29.50	0.16	0.53
	29.65	29.65	29.68	29.44	29.63			
	29.52	29.37	29.49	29.26	29.32			
Mean	29.54	29.45	29.66	29.37	29.51			
SD	0.10	0.42	0.16	0.09	0.16			
CV%	0.34	1.43	0.54	0.32	0.56			

[a]: Standard deviation
[b]: Coefficient of variation

Table 4. Precision test for the cytomegalovirus UL80.5 gene in high concentration sample. Numbers represent cycle threshold (Ct) values.

	Intra Assay					**Inter Assay**		
Parameters	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV%**
	28.17	27.95	28.20	28.19	28.21	28.28	0.52	1.83
	28.37	28.22	28.04	27.85	29.50			
	27.56	28.13	28.41	27.96	29.37			
Mean	28.03	28.10	28.22	28.00	29.03			
SD	0.42	0.14	0.19	0.17	0.71			
CV%	1.51	0.49	0.66	0.62	2.45			

[a]: Standard deviation

[b]: Coefficient of variation

Curve variability analysis

For gene UL34, the mean Ct for the points 9.65×10^5, 9.65×10^4, 9.65×10^3, 9.65×10^2, 9.65×10^1 were, respectively: 20.76, 24.08, 27.59, 31.27 and 34.74. Mean SD between curve points was 1.23, and CV was 4.55. For the gene UL80.5, mean Ct for the same points were 19.55, 22.89, 26.32, 30.03 and 33.52, respectively. Mean SD was 1.21 and CV was 4.72.

Interlaboratorial reprodutilitity

Results of the in-house test in comparison with the commercial m2000 equipment (Abbott Laboratories, USA) is presented in Table 5. Figure 2 shows

the relationship between the results found by the m2000 equipment and the in-house test.

Table 5. Comparison between results of patient samples. Numbers represent concentrations of cytomegalovirus DNA in plasma (IU/mL), expressed in log10, otherwise stated.

Sample	**m2000**	**In-house test**	**Log difference**
1 to 10	Undetected	Undetected	No difference
11	2.14	2.67	0.53
12	1.71	Undetected	Undetected
13	1.8	Undetected	Undetected
14	1.7	2.74	1.04
15	1.78	2.79	1.01
16	1.67	Undetected	Undetected
17	2.58	3.04	0.46
18	2.69	3.23	0.54
19	1.74	Undetected	Undetected
20	2.43	3.09	0.66
21	2.99	3.32	0.33
22	3.4	3.75	0.35
23	3.06	3.37	0.31
24	3.94	4.41	0.47
25	3.85	4.37	0.52

26	4.25	4.66	0.41
27	4.31	4.70	0.39
28	3.48	3.94	0.46
29	3.94	4.31	0.37
30	3.98	4.23	0.25

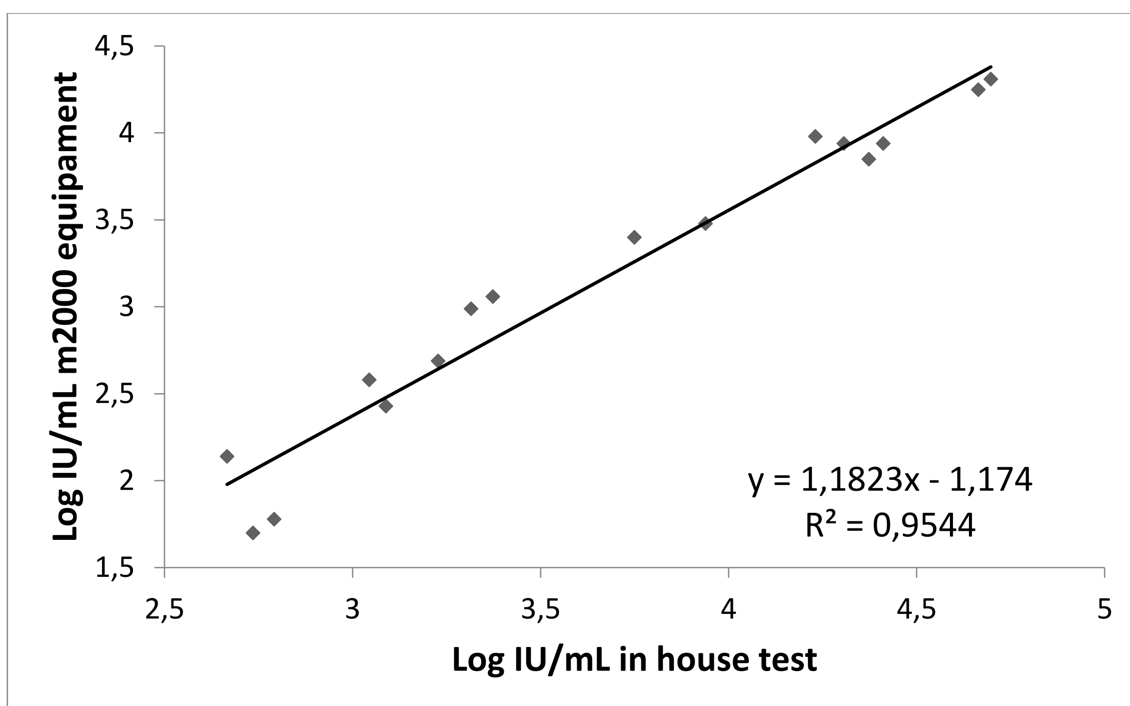


Figure 2. Comparison between results of patient's samples in m2000 equipment and the in house test.

Economic study by Cost-Minimization Analysis (CMA)

The total cost of testing per patient for the in-house qPCR assay was USD 67.25 for testing, taken into consideration that a single sample is tested. Cost decreases as the number of samples increased per run: for 10 samples the unitary cost was USD 20.52; for 30, 50 and 90 samples, cost was USD 16.97, USD 16.26 and USD 15.79, respectively.

For the assay performed on the m2000 RealTime System Equipment (Abbott Laboratories, USA) the unitary cost per exam was USD 449.42 for a single sample; for 10 samples, it was USD 102.19; and for 30, 50 and 90 samples: USD 76.47, USD 71.32 and USD 67.89, respectively.

Cost of the test currently in use at the hospital was USD 132.43 for a single sample; for 10 samples: USD 38.37; and 30, 50 and 90 samples, costs were USD 31.32, USD 29.91 and USD 28.97, respectively.

Regarding this analysis it was observed that, in the comparison between the in-house and m2000 RealTime assays there was a cost reduction of USD 382.17, USD 81.67, USD 59.50, USD 55.06 and USD 52.10 for 1, 10, 30, 50 and 90 samples, respectively. In the comparison between the in-house assay versus the kit used in the hospital, cost reduction are of USD 65.18, USD 17.85, USD 14.35, USD 13.64 and USD 13.18. The test currently in use in the hospital was most economically advantageous than the m2000 RealTime assay: USD 316.99, USD 63.82, USD 45.15, USD 41.42 and USD 38.92. In the sensitivity analysis for 10 samples being tested at the same time, the final cost of examinations ranging from 5–10% of the salaries of the professionals involved was USD 20.77–21.02, USD 38.62–38.88, USD 129.96–130.18 for the in-house test, commercial kit and m2000, respectively. The cost considering a 5%–10% increase in reagents was USD 21.24–21.97, USD 40.03–41.65, USD 132.83–135.92 for the in-house test, commercial kit and m2000, respectively. The cost minimization percentage and the economic impact of the exam cost variation are presented in Figure 3.

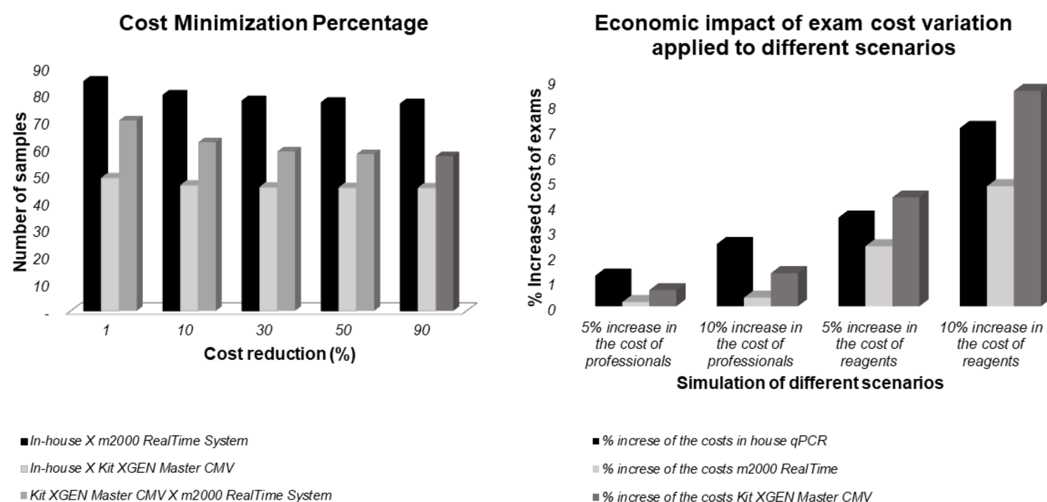


Figure 3. Cost minimization percentage and the economic impact of exam cost variation in different scenarios.

From December 2016 to December 2017, 300 patients were transplanted for kidney in the hospital. These patients need weekly evaluation for CMV in the three months after transplantation with a total of 12 samples approximately. In a scenario of 10 to 30 samples per day of test the minimization of costs goes from USD 64,260 (10 samples) to USD 51,660 (30 samples) per year using the in-house assay compared to the test currently being used and when contrasted with m2000 RealTime assay the cost minimization are USD 294,012 (10 samples) and USD 214,200.00 (30 samples).

DISCUSSION

For CMV infection and disease, molecular tests are currently considered the gold-standard for diagnosis and monitoring (23–26). However, molecular diagnosis has some limitations, which have been addressed in this study. The

first issue is variation between PCR quantification by different laboratories, especially related to the size of the amplicons reported in previous articles (27,28). In order to reduce such variabilities, primers and probes were chosen for amplicons of 98 and 105 bp. Naegele *et al.* (27) suggested in their work the standardization of amplicon size to 100 bp, and they also discuss the better reproducibility of tests using redundant targets, while others pointed out to dual targets as a better possibility (20,27–30). Another point raised in previous studies is that due to the high qPCR test sensitivity and the lack of clear cut-off to determine the need for antiviral treatment, the use of qPCR could increase considerably the number of patients treated without a real need for it. To reduce this possibility, plasma was chosen in this study as a matrix for the test, since previous works has demonstrated that in plasma the virus probably presents itself in fragmented form, while in whole blood the presence of fragmented and intracellular forms increase viral load and the chances of detection of latent infection (25,31,32).

Regarding test optimization, although several articles showed differences in results when international standard are tested with different reagents and methods (12,28,32,33), none had reported non-functioning with some reagents such as Platinum® Quantitative PCR SuperMix-UDG reagent (Applied Biosystems, USA), as observed in this study. The causes for non-functioning are uncertain, but when analyzing the reagent labels for possible causes it was observed that Platinum® Quantitative PCR SuperMix-UDG reagent (Applied Biosystems, USA) and GoTaq Probe qPCR Master Mix (Promega, USA) differ by the presence of UDG. TaqMan® Gene Expression Master Mix (Thermo Scientific, USA) also uses UDG which would not explain the non-amplification of

the primary standard, leading to believe that the problem could be in the buffer components (information that is not listed in detail in any of the package inserts), or in the concentration of the reagents (information contained only in the Platinum® Quantitative PCR SuperMix-UDG reagent package). Still on the optimization, it was observed that the efficiency of the multiplex reaction is within the ideal range, close to 90%.

Our in-house test demonstrated considerable sensitivity, as shown by the limit of detection and quantification of 60.26 IU/mL, being similar to those presented in the package insert of Abbott m2000 (31.20 IU/mL for both parameters). The difference observed in this value is probably due to the difference in the extraction methodologies and the reagents used for the tests, as well as the fact that the equipment m2000 performed the tests in a totally automated way, excluding operator-dependent bias (28,32). Also, it is important to observe that the thresholds to start therapy reported in recent studies are up to 2,000 IU/mL, so the difference in the LOD and LOQ are not clinically relevant (25). In addition, it was observed that the test was specific for CMV, since it did not cross-react with other Human Herpesviruses, nor did it show BKV amplification, which is of clinical importance in immunosuppressed as well as transplanted patients. Intra-assay and inter-assay evaluation showed high repeatability and reproducibility of the assay for both low and high concentrations, with CV results of < 5% in all readings.

Regarding inter laboratory reproducibility, it was observed that the 10 samples below the LOD of the m2000 equipment were also below the LOD of the test, a result already expected since our limit of detection is numerically higher than that of the equipment. Of the 20 tests between low and high

samples, five were below our LOD, of these two samples amplified, however with a quantitative CMV DNA log difference of > 1. Of the 20 samples amplified in the m2000 equipment, 80.0% (16/20) amplified in the test. The test failed to amplify one of the samples, which was quantified by the m2000 equipment at 62 IU/mL, close to the LOD (a concentration that is not clinically relevant). Similarly, other amplification failures occurred with samples with concentrations below the LOD. From the 16 samples quantified by the test, it was possible to observe an r^2 of 0.9544 when compared with the m2000 results. Evaluating the log difference of the test with m2000, it was found that 62.5% (10/16) had a difference < 0.5 log and that 87.5% (14/16) had a difference < 0.7 log. The mean log difference was 0.51, which is similar to what has been described in other studies (16,34–36). This difference of results found in the study is explained by the difference between the protocols used for DNA extraction, one of them being automated and the other semi-automated. Besides, the difference can be related to the reagents used in each of the tests and in the fact that the automation of the test reduces the operator-dependent factors differently of the in-house test (28,32). In addition, it is important to emphasize as a limitation of this test the fact the standard used in the test was diluted in TE for calibration of our secondary pattern, however in the 3 days of the sensitivity test the pattern did not show amplification for the 2.5×10^2, 2.5×10^1 and 2.5×10^0, as well as a curve with efficiency below 90%. Besides that, Jones *et al* evaluated the commutability of the WHO IS and reported the use of the buffer as an alternative to plasma (37).

Regarding the WHO international standard, it is important to note that although it has improved the harmonization of the CMV tests, recent studies are

finding variability that were close to 0.5 log IU/mL, yet it was not possible to state that commutability between tests was reached. Recent studies reported difficulties, such as differences in DNA quantification using the same methodology but with different extraction method until different behavior between the standard and samples because of the presentation of CMV (27,28,32,33,37–39).

The validation presented in this paper showed that the in-house assay could be considered a diagnostic equivalent to the automated test, considering its precision, analytical sensitivity and specificity. In order to evaluate the economic impact of this test a minimization cost analysis was done. The cost analysis performed shows that the in-house qPCR assay was, in median, 45.8% (range 45.5–49.2%) cheaper than the test currently used in the hospital, when compared in-house versus m2000 assay using different scenarios the median percentage of cost minimization was 77.8% (range 76.74–85.0%). A median reduction of 77.8% of the costs with the in-house method in relation to m200 RealTime System represents a considerable minimization of costs, especially for a budget adjustment and better distribution of resources in the clinical laboratory. It is important to note that this significant difference in value between exams makes it possible to use in-house assays in centers with low demand or limited budgets. In addition, it is emphasized that in case of transplanted patients, post-transplant CMV monitoring is performed weekly, which ends up burdening health plans and systems. In this context, in-house molecular testing becomes a less expensive and equivalent alternative.

The CMA applied in this study is a tool used in pharmacoeconomics when comparing health strategies most economically advantageous and it is essential

for decision-making. It was observed, in the present study, an economic projection that varies from USD 51,660 to USD 294,012 with the in-house test when compared to the commercial kit and m2000, respectively.

Researchers suggest that an economic analysis should be performed, because the greater the progress of medicine, the higher the cost to obtain further improvements. Therefore, it is necessary to decide what is the best way to spend such resources and to use them rationally (40,41). Noteworthy, simulations were considered in different scenarios addressing increased costs with professionals and reagents to verify the data robustness and reliability. It was found that when there is a 5% and 10% increase in the salaries of the professionals who perform the tests, there is a greater increase in the cost of in-house PCR, however, the final price is lower than for the other tests. Nevertheless, when there is a variation in reagent costs by 5% and 10%, there is a considerable increase in the three methodologies, even though the commercial kit had the greatest impact, but still the final value of in-house PCR remains more advantageous.

This study has some limitations, the main one being the lack of clinical data, however a court of patients is being evaluated and the results will be presented in future studies. In addition, as noted above in this section, the primary standard was diluted in TE rather than plasma due to the unsuitable results presented above. Besides that, the economic data presented in this study cannot be interlaboratory generalized due to the variability between different molecular test designs and their costs. Another point to emphasize is the impact of the fluctuation of the US dollar on imports (currently USD 1 equals 4.1

Brazilian Reais vs. 3.2 Brazilian Reais in the study period), which increases the cost of reagents.

CONCLUSION

In this study, we performed the analytical validation of an in-house qPCR assay for cytomegalovirus compared to the automated test of the m2000 equipment. The test was sensitive, specific and precise, and showed good linearity with the m2000 equipment. The results show that the in-house methodology is diagnostically equivalent to m2000, since the differences between the tests occur close to the LOD and LOQ, which are not clinically relevant once the thresholds for the beginning of treatment, presented in the last studies are above 2000 IU/mL. Therefore, a cost minimization analysis was performed pointing out the in-house test as a more economically advantageous alternative, allowing laboratories with budget constraints to offer this diagnostic tool. The cost minimization presented by the in-house test was maintained after the sensitivity analysis, demonstrating the robustness of the findings. Besides these results it is important to notice that it has been recently discovered that CMV can be found in diverse forms in different matrices, which impacts on the size of the primers and probes and the virus dynamics in the infected patients. The total commutability was not achieved but today it is possible to perform an in-house test and the results are comparable with automated platforms as shown in this study.

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CONFLICTS OF INTEREST

Declarations of interest: none.

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Supplementary material

Cost minimization analysis and validation of a cytomegalovirus quantitative in-house real time PCR assay in relation to m2000 RealTime System

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Primers and probes used in this study targeted the genes UL34 and UL80.5. Sequences are shown below.

Genes	Sequences (5’–3’)
UL34 (105 bp)	F- TGAACTTCATCATCACCACCCGAGACT R- CCTTGTACGCTTTGGAAATCGAGCCTG P- FAM-CGACGATTCAGTCCTGCGAGCC-QSY
UL 80.5 (98 bp)	F- CGGCTAGTGTCGTGTTAGC R- CACAAAAATCCGCCGATTCAGATC P- VIC-AAGCCGCCGCAGCTTCCCAG-QSY

The sequence of the plasmid used for the standard curve is shown below. Primers and probes are shown in yellow for the UL34 gene, and in green for the UL80.5 gene:

CCCCACCGCCGTCGTCGTCATGAACTTCATCATCACCACCCGAGACTTCT
CCAACGACGATTCAGTCCTGCGAGCCGCCGAGATGCGTGACAACGTGGC
AGGCTCGATTTCCAAAGCGTACAAGGGCACGGTACGCGCCGAAGGCTTTT
TGGCAGGTTCTTCTTCCTGCCCGGCTAGTGTCGTGTTAGCCGCCGCTGCT
GCCCAAGCCGCCGCAGCTTCCCAGAGCCCGCCCAAAGACATGGTAGATC
TGAATCGGCGGATTTTTGTGGCTGCGCTCAATAAGCTCGA

4.2 ARTIGO 2

“Clinical validation of an in-house quantitative real time PCR assay for cytomegalovirus infection using the 1st WHO International Standard in kidney transplant patients”

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Clinical validation of an in-house quantitative real time PCR assay for cytomegalovirus infection using the 1st WHO International Standard in kidney transplant patients

Running Title: Clinical validation of an in-house qPCR for CMV

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ABSTRACT

Introduction: Cytomegalovirus (CMV) is one of the most common agents of infectious diseases in solid organ transplant patients, with significant morbidity and mortality. **Objective:** This study aimed to establish a threshold for initiation of preemptive therapy in a cohort of kidney transplant patients with an in-house quantitative real time PCR assay (qPCR), which was calibrated using the 1st WHO International Standard. **Study design:** This was a prospective cohort study conducted in 2017 in a single kidney transplant center in Brazil. Clinical validation was performed by comparing in-house qPCR results against Pp65 CMV antigenemia. ROC curve analysis was performed to determine the ideal threshold for preemptive therapy using the qPCR test. **Results:** 232 samples from 30 patients were tested with both antigenemia and qPCR, from which 163 (70.26%) samples showed concordant results (Kappa coefficient: 0.435, $p<0.001$ and Spearman correlation: 0.663). The median number of days for the first positive result was 50 (range, 24–105) for antigenemia and 42 (range, 24–74) for qPCR ($p<0.001$). ROC curve analysis revealed that at a threshold of 3,430 IU/ml (Log 3.54), qPCR had a sensitivity of 97.06% and a specificity of 74.24% (AUC 0.92617 ± 0.0185, $p<0.001$), in the prediction of 10 cells/10^5 leukocytes by antigenemia and physician's decision to treat. **Conclusions:** CMV Pp65 antigenemia and CMV qPCR showed fair agreement and a moderate correlation in this study. The in-house qPCR revealed to be an accurate method to determine CMV DNAemia in kidney transplant patients, resulting in positive results weeks before CMV Pp65 antigenemia.

KEYWORDS: Cytomegalovirus; qPCR; antigenemia; threshold; preemptive therapy.

BACKGROUND

Cytomegalovirus (CMV) (Order *Herpesvirales*, Family *Herpesviridae*, Subfamily *Betaherpesvirinae*, Genus *Cytomegalovirus*, Species *Human betaherpesvirus 5*) is one of the most relevant causes of infection in transplant organ recipients, resulting in significant morbidity and mortality [1]. Infection can originate from the transplanted organ or more commonly due to reactivation of previous (latent) CMV infection in the recipient [2].

Most patients at risk of CMV infection/disease are monitored with diagnostic tests aiming for an early detection of CMV infection, in the preemptive strategy. Laboratory monitoring for preemptive therapy was in early years performed with antigenemia. However, molecular assay have replaced antigenemia to become the gold-standard for CMV diagnosis and monitoring [3]. However, due to large inter-assay variations, no universal consensus has been reached on the threshold to initiate therapy against CMV [3–5].

In this scenario, this study aimed to establish a threshold for initiation of preemptive treatment against CMV in a cohort of kidney transplant patients. In addition, the study compared the performance of Pp65 CMV antigenemia and a novel in-house quantitative real time PCR assay, which was calibrated using the 1st WHO International Standard for Human Cytomegalovirus.

MATERIAL AND METHODS

Patients and samples

This was a prospective observational cohort study conducted from January to April 2017. All adult (older than 18 years-old) kidney transplant patients being taking care of due to kidney transplantation at Santa Casa de Misericórdia de Porto Alegre, Brazil, were considered for inclusion in the study. Patients were followed weekly for at least three months after kidney transplantation. Plasma samples for CMV qPCR tests were collected weekly using 4 ml EDTA. Samples were centrifuged at 1300 xg for 15 min for plasma separation and stored at -80ºC until the time of nucleic acid extraction.

As part of the routine hospital care, patients received anti-CMV therapy based on antigenemia results, based on a threshold of 10 cells/10^5 leukocytes; patients presenting with less than 10 cells/10^5 leukocytes but showing symptoms attributable to CMV disease were also put on anti-CMV treatment.

Data Collection

Clinical and demographic data were collected for all patients who entered the study. These variables included underlying diseases, induction therapy, regimen of immunosuppression, and CMV serology for both donors and recipients.

CMV Pp65 Antigenemia

CMV antigenemia test was performed using the CMV Brite™ Kit (IQ Products, The Netherlands), as part of patients' routine monitoring for CMV infection.

Quantitative In-house real time PCR assay

DNA was extracted from plasma samples with Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, USA) following the manufacturer's instructions.

Primers and probes used in this study were described by Ho and Barry and the sequences are shown in the supplementary material with some modification in the probe design [6].

The standard curve used in the assay was obtained with a plasmid with a sequence of the CMV genome (supplementary material), which has been validated with the 1st WHO International Standard for Human Cytomegalovirus (WHOIS), generating a conversion factor for international units. The detailed qPCR description and the curve parameters are shown in the supplementary material. Only results above the limit of quantitation and detection were considered positive.

Statistical analysis

The comparison between the tests was performed using the Cohen's kappa coefficient and Spearman's correlation coefficient; results were interpreted according to Altman *et al* and Akoglu *et al* respectively [7,8]. Comparison of medians of antigenemia and qPCR results between patients who were asymptomatic and symptomatic was made using the T-test for independent samples. A ROC curve analysis was performed to determine the threshold to initiate preemptive therapy. Statistical analyses were performed with SPSS Software (Statistical Package for the Social Sciences), version 18.0.

Ethical aspects

The ethics committees of the Universidade Federal de Ciências da Saúde de Porto Alegre and the Santa Casa de Misericórdia of Porto Alegre approved the present study, in accordance with the precepts of the Declaration of Helsinki by the following protocol numbers: 1.820.875 and 1.885.683. Written consent was obtained for all patients before entering the study. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

RESULTS

From December 2016 to December 2017, 300 kidney transplant procedures were performed in the hospital, from which 51 patients from January to April participated in the study. Twenty-one patients were excluded from the study due to poor adherence to the collection of laboratory exams or missing consultations. The final study population consisted of 232 plasma samples from 30 patients (average of 7.7 samples per patient, ranging from 5–14). Patient demographic characteristics are presented in Table 1.

Patients' Characteristics	
Recipient	**Frequence (%)**
Sex	
Male	60
Age (years)	
Median (Range)	53.5 (21–71)
Race	
Caucasian	83.3
Cause of ESRD	
Unknown	26.7
Polycystic kidneys	20
Focal segmental glomerulosclerosis	13.3
Type 2 diabetes mellitus	13.3
Type 1 diabetes mellitus	6.7
Systemic lupus erythematosus	6.7
Systemic arterial hypertension	3.3
Berger's disease	3.3

Alport's disease	3.3
Chronic glomerulonephritis	3.3
PRA class I (%)	
0	60
1–49	26.7
50–79	10
≥ 80	3.3
PRA class II (%)	
0	40
1–49	33.3
50–79	23.3
≥ 80	3.3
DSA quantity (%)	
1	8
Induction therapy	
Tacrolimus + Mycophenolate sodium + Steroids	100
Antithymocyte globulin	40
Basilixumab	60
Hemodialysis until 1st week after transplantation	
Yes	40
Donor	
Sex	
Male	66.7

Age	
Median (Range)	49.5 (1–70)
Donor/ Recipient serostatus for CMV infection	
D+ / R+	53.3
D- / R+	33.3
D+ / R-	6.7
D- / R-	3.3

Legend: D: donor, DSA: donor specific antibody, ESRD: end stage renal disease, HLA: human leucocyte antigen, PRA: panel reactive antibodies, R: recipient, SD: standard deviation.

Table 1. Demographic characteristics of patients evaluated in this study.

Of the 232 samples from 30 patients evaluated in the study, 102 (44%) were negative for both CMV qPCR and antigenemia, 61 (46.9%) were CMV positive for both methods, 68 samples (52.3%) were CMV positive by qPCR only, and 1 sample (0.8%) was only CMV positive by antigenemia. qPCR and antigenemia tests were concordant in 163 samples (70.3%) (Kappa coefficient test 0.435; $p<0.001$); the Spearman correlation test was 0.663 ($p<0.001$).

Of the 69 discordant samples between qPCR and antigenemia, it was possible to observe that 54 (78.3%) of the discordant results occurred just before (median of 12 days, range, 0–25 days) or soon after (median of 9 days, range, 0–28) antigenemia became positive or negative, respectively. Figure 1 shows the comparison between the first positive sample and first negative sample, for each of the tests. Regarding the 15 samples (21.7%) that showed positive results for qPCR in patients with negative antigenemia, all patients presented negative qPCR during the following weeks, and the qPCR results

varied from Log 2.79 IU/ml to Log 3.97 IU/ml. The only case of positive antigenemia (1 cell/10^5 leukocytes) with negative qPCR tests occurred in a patient who presented with DNAemia in previous weeks, and the patient became negative after a few weeks for both antigenemia and qPCR tests.

INSERT FIGURE 1

During the study, of the 30 patients included, only five did not present positive results for any of the methods used. Among the 25 patients with positive tests, 21 (84%) had at least one positive result for both tests and four (16%) had only qPCR positivity. The Kappa concordance coefficient for patient concordant results was 0.636 ($p<0.001$). The median number of days for the first positive result to occur was 50 (range, 24–105 days) for antigenemia and 42 (range, 24–74 days) for qPCR ($p<0.001$). Of these 25 patients, 17 were treated with intravenous ganciclovir for CMV infection or disease, 4 had decreased immunosuppression without the need for ganciclovir treatment, and the other 4 patients received no intervention once the antigenemia was negative and the physician did not have access to qPCR results. Of the 25 patients with a positive result, 11 were symptomatic but only 3 (12%) developed CMV disease, 22 (88%) had only infection. The symptoms related to CMV were: leucopenia in 7 (28%), thrombocytopenia 6 (24%), diarrhea 3 (12%) and oral mucosal lesions 1 (4%). Pancytopenia was observed in 1 (4%) case of CMV disease. A significant difference was found between the median number of cells in patients who were symptomatic and patients who were asymptomatic, the median was respectively 7.0 cells/10^5 leukocytes (range 1 to 580 cells/10^5 leukocytes) and 3.0 cells/10^5 leukocytes (range 1 to 48 cells/10^5 leukocytes) ($p=0,021$). The assessment of the qPCR median results also showed a

significant difference between the outcomes of patients who were symptomatic and asymptomatic, respectively, being 15,539.02 IU/ml (range 528.66 to 605,059.08 IU/ml) and 3,490.12 IU/ml (range 166.04 to 486,978.25 IU/ml) ($p < 0.001$). Of the 5 (16.7%) patients who underwent prophylactic therapy, 5 (100%) had detectable DNAemia but none developed disease. Of the 25 (83.3%) patients on preemptive therapy, 20 (80.0%) developed DNAemia and 3 (12.0%) developed disease.

Evaluating the infection and disease within the serostatus groups, it was possible to observe that of 16 (53.3%) D+/R+ patients, 15 (93.8%) presented DNAemia and 2 (12.5%) of these developed disease; of the 10 (33.3%) patients in the D-/R+ group 9 (90.0%) had DNAemia and 1 (10.0%) of them developed disease; of 2 (6.7%) patients in the D+/R- group 1 (50.0%) had DNAemia; the only patient in the D-/R- group did not present DNAemia.

Figure 2 shows the performance of qPCR test in the prediction of significant results by CMV antigenemia and physicians intention to initiate anti-CMV therapy. Three thresholds were tested: 2,750 IU/ml (Log 3.44), 3,430 IU/ml (Log 3.54) and 3,650 IU/ml (Log 3.56), qPCR had a sensitivity of 100%, 97.06% and 91.18% and a specificity of 71.72%, 74.24% and 75.25% respectively (AUC 0.92617 ± 0.0185, $p<0.001$), in the prediction of detecting 10 cells/10^5 leukocytes by antigenemia and physician's decision to treat.

INSERT FIGURE 2

When the agreement between the positive antigenemia tests and the qPCR tests considered positive from the cutoffs established was analyzed, Kappa correlation coefficients of 0.588 (Log 3.44), 0.604 (Log 3.54) and 0.557 (Log 3.56) were observed.

DISCUSSION

Despite advances in the diagnostic field, CMV infection still results in high rates of morbidity and mortality among solid organ transplant recipients [1]. In this prospective cohort of kidney transplant patients, a high CMV infection rate (83.3%) was observed, while CMV disease occurred in 10.0% of patients. A study performed in the same institution in 2004 using CMV antigenemia as a diagnostic tool observed a lower infection rate (60.0%), whereas the disease frequency was higher 38.4% [9]. In a study carried out in another hospital in the same city in Brazil, with a composition of patients that was similar to that of the present study, the incidence of CMV infection was 53.3% [10]. This cohort was characterized by an elevated seroprevalence of CMV infection in both donors and recipients, and by a limited proportion of patients on universal anti-CMV prophylaxis (16.6% of patients only in, comparison to 50.0%, in the study by Franco et al) [10–12]. Another study conducted in Brazil in a low-risk population of kidney transplant recipients found an incidence rate of 69.6% using antigenemia and qPCR methodologies [13], yet a cohort study performed in heart transplant recipients found a rate of 93.3% incidence [14]. The incidence rates found in Brazil are similar to studies in Japan (70.8%) [15] and India (73.7%) [16], but differ from countries such as Korea, where the literature shows rates of 30–40% [17–20], Finland of 27% [21] and in the USA, where a rate of 27% was found in a pediatric kidney transplant population [22].

The comparison between the two diagnostic tests performed in this study showed a concordance between the results of 70.3%, in agreement with previous studies that demonstrated concordances ranging from 66.6–94.3% [10,14,17–19,22–24]. However, most of these studies were performed before

the advent of the WHOIS, as well as before the knowledge of factors related to the presentation of the virus in different biological matrices [5,25,26]. These factors drastically influence the reproducibility, sensitivity and specificity of molecular tests. Kamei *et al* found agreement between 87.4% methodological results using a WHOIS calibrated assay in liver transplant patients [24]. In the Kappa test it was possible to observe a fair agreement between the tests, which was also seen by Franco *et al,* Rhee *et al* and Choi *et al*. In the studies of Rha *et al* and Kwon *et al* strong concordances were found 0.61 and 0.66, respectively [10,17,20,22,27]. The correlation between the tests was fair when the threshold of Log 3.44 and Log 3.56 were considered for positive results and moderate when Log 3.54 was used as threshold. The agreement between the tests becomes good when evaluated positivity among the patients, the same thing happened in previous studies [10]. When performing an analysis to evaluate the correlation between the assays, we found a moderate relationship, corroborating the results found by Ishii *et al*, Kamei *et al* and Rhee *et al* [15,18,24]. This moderate relationship between tests could be explained by the fact that antigenemia is a semi-quantitative technique, operator-dependent, and qPCR is a quantitative technique that allows the automation of several steps. In addition, analyzing the discordant results it is possible to verify that most of them are explained by the greater sensitivity of the molecular assays when compared to the antigenemia, since the positive results in the qPCR turned positive and negative more than a week before and after the CMV antigenemia test [17]. The median number of days for positivity of antigenemia was 50, and for qPCR was 42 days. This result is similar to that found by David-Neto *et al* in a double-blind study to determine the cut-off point for initiation of treatment by

the preemptive strategy in low-risk kidney transplant patients [28]. It is important to note that most of the patients in this study are considered low risk (D+/R+).

After nearly ten years of the launch of the WHOIS a consensual threshold for treatment of cytomegalovirus has not been defined yet [3,29]. The third international consensus on the management of cytomegalovirus in patients with solid organ transplants indicates that it is desirable for the centers to define by themselves a threshold, taking into account the type of assay, type of biological sample and risk factors of the patients [3]. In order to balance the sensibility and specificity of the threshold, 3,430 IU/ml (Log 3.54) was chosen to initiate the therapy if 10 cells/10^5 leukocytes on antigenemia and physicians' decision were used as the gold-standard. The sensibility of the threshold established in this study was very high (97.06%) but specificity was not optimal (74.2%) which could be explained by the samples that only were positive in qPCR, but it is important to emphasis that most of the results occurred days before or after positive antigenemia results. Previous plasma studies used different threshold values for low-risk patients, one including 3,983 IU/ml threshold (log 3.60 IU/ml), and another 2,750 IU/ml (log 3.44 IU/ml) for low-risk patients, and 1,500 IU/ml (log 3.18 IU/ml) for high-risk patients [30,31]. Considering patients with positive qPCR results who had negative antigenemia (4/30) in this study, only two of the patient reached the threshold point of Log 3.54; therefore, specificity was 93.3%.

This study has some limitations, the main one being the small number of patients investigated; twenty-one patients were excluded from the study due to poor adherence to the collection of laboratory exams or missing consultations and the fact that the population is composed mostly of low-risk patients, not

allowing to generalize our threshold values to other patient populations. However, we emphasize that this occurred due to the high seroprevalence of CMV infection in this population.

In conclusion, the two CMV diagnostic tests used in this study, qPCR and antigenemia, showed a fair correlation. Recent knowledge on the relevance of viral kinetics allows for the development of increasingly sensitive molecular tests that improve evaluation of CMV DNAemia in patients, with positive results ahead of what was previously seen with antigenemia only. However, this high sensitivity requires a careful clinical evaluation of the threshold for the initiation of treatment, in order to avoid unnecessary treatment of these patients. Here we demonstrate the optimal threshold value for using an in-house qPCR in the management of CMV infection in kidney transplant patients, using the WHOIS as a standard. More studies using qPCR calibrated with the WHOIS are needed so that it is possible to compare thresholds, searching for one that can be extrapolated to populations of patients with different risks.

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Conflict of Interest Declaration:

None declared.

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Hidalgo, M. Sánchez, M.A. Gentil, C. Bernal, J.M. Sobrino, M.J. Rodríguez-Hernández, E. Cordero, Viral load, CMV-specific T-cell immune response and cytomegalovirus disease in solid organ transplant recipients at higher risk for cytomegalovirus infection during preemptive therapy, Transpl. Int. 27 (2014) 1060–1068. https://doi.org/10.1111/tri.12378.

FIGURES

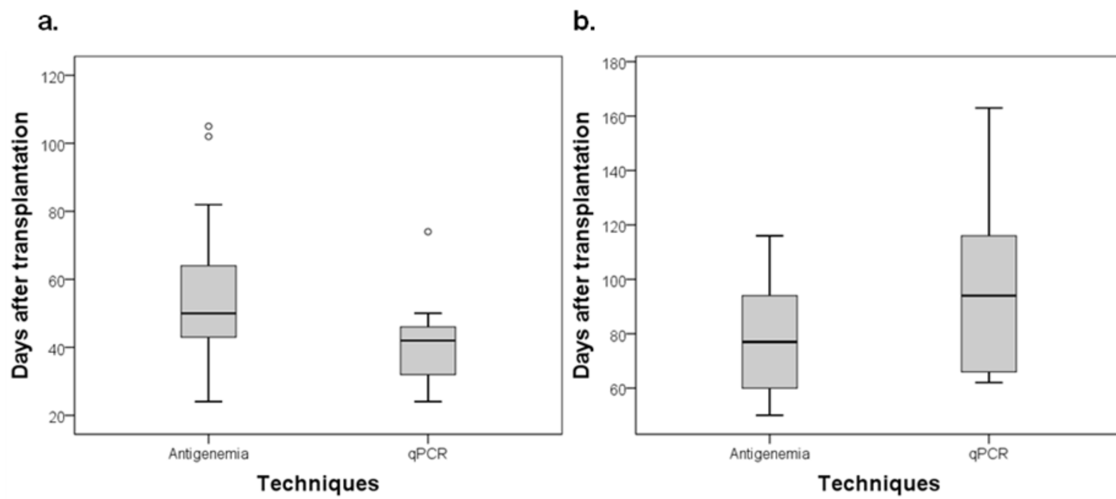

Figure 1. Comparison between antigenemia and qPCR regarding (a) First positive sample for CMV for antigenemia and qPCR; and (b) First negative sample for each of the tests.

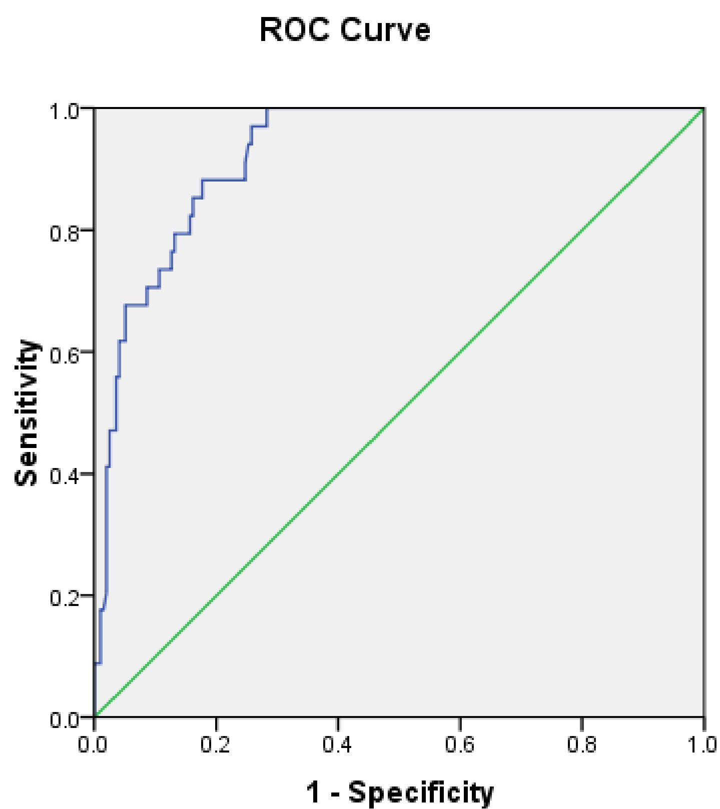

Figure 2. ROC curve analysis for the performance of CMV antigenemia and

physician's intention to initiate anti-CMV therapy. The threshold points evaluated were: 2,750 IU/ml (Log 3.44), 3,430 IU/ml (Log 3.54) and 3,650 IU/ml (Log 3.56), qPCR had a sensitivity of 100%, 97.06% and 91.18% and a specificity of 71.72%, 74.24% and 75.25% respectively (AUC 0.92617 ± 0.0185, $p<0.001$), in the prediction of detecting 10 cells/10^5 leukocytes by antigenemia and physician's decision to treat.

Supplementary material

Clinical validation of an in-house quantitative real time PCR assay for cytomegalovirus infection using the 1st WHO International Standard in kidney transplant patients

Authors: Cassia F.B. Caurio[a,b], Odelta S. Allende[a], Roger Kist[a,b], Kênya L. Santos[b], Izadora C.S. Vasconcellos[a,b], Franciele P. Rozales[a], Daiane F. Dalla Lana[b], Bruno M. Praetzel[b], Ana Paula Alegretti[c], Alessandro C. Pasqualotto[a,b]

[a]Santa Casa de Misericórdia de Porto Alegre, Porto Alegre, Brazil.

[b]Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil.

[c]Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

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The PCR reaction was performed to a final volume of 20 µL using 4 µL of ultrapure water, 3 µL of extracted DNA, 0.4 µM of each primer, 0.25 µM of each probe, 10 µl of GoTaq Probe qPCR Master Mix (Promega, USA) and 0.4 µL of carboxy-X-rhodamine (CXR) in a 1:50 dilution. Parameters for qPCR are shown in Figure S1.

Primers and probes used in this study targeted the genes UL34 and UL80.5. Sequences are shown below.

Genes	Sequences (5'–3')
UL34	F- TGAACTTCATCATCACCACCCGAGACT
	R- CCTTGTACGCTTTGGAAATCGAGCCTG
	P- FAM-CGACGATTCAGTCCTGCGAGCC-QSY
UL 80.5	F- CGGCTAGTGTCGTGTTAGC
	R- CACAAAAATCCGCCGATTCAGATC
	P- VIC-AAGCCGCCGCAGCTTCCCAG-QSY

The sequence of the plasmid used for the standard curve is shown below. Primers and probes are shown in bold for the UL34 gene, and in underline for the UL80.5 gene:

CCCCACCGCCGTCGTCGTCA**TGAACTTCATCATCACCACCCGAGACT**TCT

CCAA**CGACGATTCAGTCCTGCGAGCC**GCCGAGATGCGTGACAACGTGG**C**

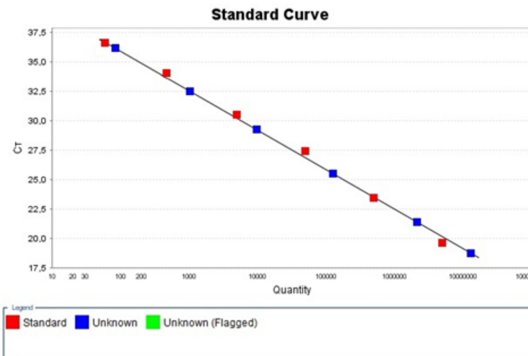
AGGCTCGATTTCCAAAGCGTACAAGGGCACGGTACGCGCCGAAGGCTTT

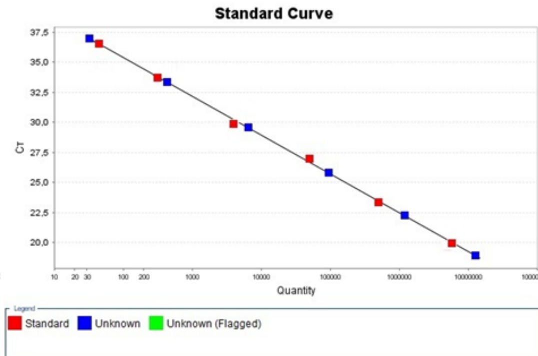
TTGGCAGGTTCTTCTTCCTGCCCGGCTAGTGTCGTGTTAGCCGCCGCTGC

TGCCCAAGCCGCCGCAGCTTCCCAGAGCCCGCCCAAAGACATGGTAGAT

CTGAATCGGCGGATTTTTGTGGCTGCGCTCAATAAGCTCGA

UL 34	UL 80.5
A) **Slope:** *-3.35* **Y-inter:** *42.63* **R²:** *0.99* **Eff:** *98.83*	B) **Slope:** *-3.55* **Y-inter:** *42.74* **R²:** *0.99* **Eff:** *91.43*





THRESHOLD: 0,21 (UL 34) e 0,12 (UL 80.5)
LIMIT OF QUANTIFICATION AND DETECTION: 60,26 IU/mL
CONVERSION FACTOR: 0,29

Figure S1. qPCR Assay Parameters

5. CONCLUSÕES

O Brasil é o segundo país com maior número absoluto de transplantes renais do mundo. Nesse contexto, o serviço de transplante renal da ISCMPA é referência não só no país, mas também na América Latina. Foram realizados 300 transplantes renais de dezembro de 2016 a dezembro de 2017 nessa instituição.

Na população de pacientes transplantados renais, a infecção e a doença por citomegalovírus ainda representam um obstáculo, como representado pelas altas taxas deste estudo: 83,3% de infecção e 10% de doença. Buscando reduzir a infecção e evolução da doença, nosso grupo realizou a validação de um ensaio *in-house* de qPCR, o qual permite melhor monitoramento pós-transplante desses pacientes.

O teste desenvolvido apresentou resultados adequados, demonstrando alta sensibilidade com um limite de detecção e de quantificação de 60.26 IU/mL, sendo, também, específico para CMV quando avaliados outros patógenos da família *Herpesviridae* e BK vírus. Quando comparado ao exame automatizado do equipamento m2000, nosso teste apresentou r^2 de 0,9544, o que demonstra a linearidade entre os testes. A diferença encontrada entre os resultados do ensaio *in-house* e do teste automatizado está de acordo com os achados de outros grupos que estudam citomegalovírus, sendo a diferença próxima de 0,5 e 0,7 log UI/mL.

No âmbito financeiro, observamos que esses pacientes necessitam de um monitoramento semanal, o que acaba por onerar, especialmente, a instituição e o Sistema Único de Saúde (SUS), portanto nosso teste vem como uma alternativa com custo inferior aos testes do mercado. O exame atualmente

utilizado pela ISCMPA tem uma mediana de custo de 45,8% maior que o teste desenvolvido neste trabalho. Ademais, observamos que a automação tem uma mediana de custo de 77,8% maior que o do teste desenvolvido. Não foi possível realizar o cálculo de custo do exame de antigenemia em virtude das alterações que foram realizadas na Santa Casa durante o período do estudo. Atualmente, a antigenemia não é mais utilizada para monitoramento dos pacientes transplantados renais, tendo sido substituída por um ensaio de qPCR comercial, o qual fez parte da avaliação econômica reportada acima.

Quanto à comparação entre as metodologias de antigenemia e de qPCR, observamos que elas apresentam uma concordância justa e correlação moderada. Grande parte dos resultados discrepantes encontrados no estudo são explicados pela sensibilidade superior do qPCR que apresentou resultados positivos mais de uma semana antes e depois da antigenemia. Essa sensibilidade é vista, muitas vezes, como uma limitação quando tratamos de CMV, devido à latência do patógeno. Para minimizar essa adversidade, optamos pelo plasma como matriz biológica e realizamos uma análise de curva ROC levando em consideração o ponto de corte já utilizado pelos clínicos na instituição para tratar a partir da antigenemia e o início do tratamento pelo médico, sendo de 3.430 UI / ml (Log 3,53) o ponto de corte estabelecido.

A população do estudo – mesmo sendo composta em sua maioria por pacientes de baixo risco D+/ R+ – apresentou alta taxa de positividade para o vírus; de 30 pacientes, 25 (83,33%) apresentaram em algum momento do estudo resultado positivo para o vírus por algum dos métodos. Desses pacientes, 21 (84%) necessitaram de intervenção, 17 deles com Ganciclovir. Observamos também que pacientes sintomáticos apresentaram contagem de

células e DNAemia significativamente superior aos pacientes assintomáticos. Os pacientes apresentaram uma mediana de 50 dias pós-transplante para o primeiro resultado positivo na antigenemia e de 42,5 dias no qPCR.

Por fim, este estudo permitiu uma aproximação entre as equipes técnicas do laboratório e os clínicos, tornando possível o diálogo sobre as dificuldades e demandas de ambos. Observamos que o desenvolvimento e aplicação de testes diagnósticos para citomegalovírus é um desafio global devido à biologia desafiadora desse patógeno. Acrescentamos a esse percalço a limitação orçamentária que encontramos em nossa instituição. Conseguimos, a partir desta parceria, desenvolver um teste com uma capacidade diagnóstica ótima, em que os resultados foram testados em nossa população e, somado a isto, com custo melhor do que o oferecido atualmente.

6. BIOGRAFIA

Graduada em Biomedicina pela Universidade Federal de Ciências da Saúde de Porto Alegre, com habilitações em análises clínicas e biologia molecular. Durante a graduação dedicou-se a iniciações científicas nas áreas de marcadores tumorais e biologia molecular de patógenos.

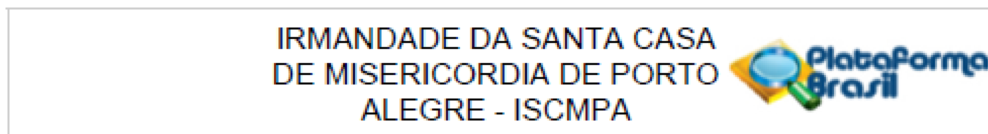
Participa ativamente das pesquisas realizadas pelo grupo de diagnóstico molecular e por imagem em medicina translacional, tendo publicado quatro artigos em dois estudos multicêntricos realizados para avaliar a epidemiologia e os testes diagnósticos para *Clostridium difficile* e *Histoplasma capsulatum* no Brasil.

Atualmente, trabalha como analista clínica e auditora interna no Laboratório de Análises Clínicas Carlos Franco Voegeli da ISCMPA e realiza mestrado no Programa de Pós-graduação em Patologia da UFCSPA. Além disso, é Bolsista de Apoio Técnico CNPq no Laboratório de Biologia Molecular da ISCMPA.

Visa permanecer no meio acadêmico, já tendo seu projeto de doutorado aprovado em 7 centros no país, bem como registrado no *Clinical Trials*. O projeto “Estudo Randomizado Aberto de Fase II Avaliando Três Regimes de Anfotericina B Lipossomal como Terapia de Indução para Histoplasmose Disseminada em Pacientes com AIDS” é um dos maiores já realizados no mundo para essa patologia.

7. ANEXOS

7.1 ANEXO I- Parecer Consubstanciado do CEP da ISCMPA



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Comparação entre os métodos de antigenemia e de PCR em tempo real para citomegalovírus em transplantados renais.

Pesquisador: ALESSANDRO COMARÚ PASQUALOTTO

Área Temática:

Versão: 1

CAAE: 61143916.7.0000.5335

Instituição Proponente: Irmandade da Santa Casa de Misericordia de Porto Alegre - ISCMPA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.820.875

Apresentação do Projeto:

O transplante renal é a principal metodologia para pacientes com prejuízos irreversíveis das funções renais. Atualmente, o Brasil é o
segundo em número absoluto de transplantes renais, sendo o citomegalovírus (CMV) o patógeno considerado de maior importância nesses
pacientes, visto que pode ser transmitido ao receptor pelo órgão transplantado ou por transfusões sanguíneas; a reativação do vírus latente também
pode ocorrer, bem como a reinfecção por outro sorotipo diferente. Esse microrganismo pode causar efeitos diretos e indiretos e está associado a
uma alta taxa de morbidade e mortalidade. O monitoramento para esse patógeno ocorre no pré-transplante por meio da sorologia, a fim de
classificar o receptor e o doador quanto à exposição prévia ao vírus. E, no pós-transplante, pelas técnicas de histopatologia, cultura, sorologia,
antigenemia e ensaios moleculares para detecção e quantificação de ácido nucleico para CMV (NAT). A antigenemia é a técnica adotada na Santa
Casa de Misericórdia de Porto Alegre para iniciar e monitorar o tratamento preemtivo; contudo, o panorama mundial indica que grande parte

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UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

laboratórios já migraram para o NAT, tendo em vista sua sensibilidade e especificidade superior. Recentemente, a Organização Mundial de Saúde
disponibilizou um Padrão de Referência Internacional, permitindo, assim, melhor padronização dos ensaios de NAT entre os laboratórios.
Internacionalmente, diversos estudos clamam pela harmonização dos padrões desse teste, o que só ocorrerá quando mais pesquisas sejam
realizadas com esse fim. Objetivo: Comparar a sensibilidade das metodologias de antigenemia e de PCR em tempo real no pós-transplante renal
para o CMV.Materiais e Métodos: Estudo de coorte prospectivo observacional unicêntrico. Serão incluídos pacientes maiores de 18 anos, indicados
para transplante renal no Hospital Dom Vicente Scherer, diagnosticados com doença renal crônica, que assinarem o termo de consentimento livre e
esclarecido (TCLE). O resultado dos exames sorológicos serão obtidos através do banco de dados, via prontuário eletrônico, uma vez que estes são
realizados na rotina pré-transplante. Uma ficha clínica será preenchida com os dados pertinentes para atender aos objetivos secundários, os dados
demográficos complementares serão pesquisados também nos bancos de dados da Instituição. No pós-transplante quatro amostras do paciente em
30, 45, 60 e 90 dias após o transplante serão analisadas, visando compara-las com os resultados obtidos da antigenemia, que também é realizada
como protocolo pós-transplante na instituição de pesquisa. Essas amostras são coletadas para os exames de rotina pós-transplante, parte delas
serão destinadas ao exame de antigenemia e o excedente ao estudo em questão.Aspectos éticos: Este projeto foi enviado para o Comitê de Ética
em Pesquisa da Irmandade da Santa Casa de Misericórdia de Porto Alegre, bem como para o Comitê de Ética em Pesquisa em Seres Humanos da
Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

Objetivo da Pesquisa:

Objetivo Primário:

• Comparar a sensibilidade das metodologias de antigenemia e de PCR em tempo real no pós-transplante renal para a detecção de CMV.

Objetivo Secundário:

Endereço: R. Profª Annes Dias,295 Hosp.Dom Vicente Scherer
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Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

• Determinar pontos de corte para o método de PCR em tempo real para CMV, em amostras de plasma, que possam diferenciar infecção de
doença; • Determinar o custo para ambos os testes e calcular custo/beneficio de seus usos;• Avaliar os fatores de risco para CMV nos
transplantados renais na Santa Casa;• Avaliar a soroprevalência de doadores e receptores de enxerto renal na Santa Casa no período de Janeiro
a Outubro de 2017;• Avaliar a incidência de infecção ativa por CMV nos primeiros três meses pós-transplante renal, por qualquer dos métodos
diagnósticos empregados;• Documentar as estratégias de prevenção e de tratamento para CMV em transplantados renais na Instituição;• Avaliar
quais os principais patógenos relacionados a co-infecções com o CMV, nesta coorte;• Avaliar os principais efeitos indiretos do CMV em pacientes
no pós-transplante renal;• Avaliar a morbidade e a mortalidade nos pacientes transplantados renais na Santa Casa, no período do estudo;•
Avaliar a prevalência de resistência aos medicamentos administrados como tratamento para CMV em pacientes transplantados renais, no período
estudado.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos referentes ao estudo estão relacionados a coleta de sangue venoso periférico, podendo gerar desconforto ao paciente, bem como
hematomas.

Benefícios:

A participação nesse estudo gerará benefício para os pacientes que farão esta mesma cirurgia no futuro, pois uma forma mais rápida e melhor de
diagnosticar esse vírus pode diminuir os problemas causados em decorrência da doença, como, por exemplo, a rejeição do órgão.

Comentários e Considerações sobre a Pesquisa:

É um trabalho interessante de prevalência em nosso meio.

Considerações sobre os Termos de apresentação obrigatória:

Apresentados e Adequados.

Conclusões ou Pendências e Lista de Inadequações:

O projeto está adequado conforme a resolução vigente 466/12, aprovado.

Considerações Finais a critério do CEP:

Após avaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer.

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA. Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 - Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_797495.pdf	19/10/2016 08:46:16		Aceito
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Cronograma	cronogramanovocep.pdf	19/10/2016 08:43:17	Cássia Ferreira Braz Caurio	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	19/10/2016 08:42:59	Cássia Ferreira Braz Caurio	Aceito
Projeto Detalhado / Brochura Investigador	projetonovo.pdf	19/10/2016 08:41:45	Cássia Ferreira Braz Caurio	Aceito
Orçamento	orcamento.jpg	14/10/2016 10:44:57	Cássia Ferreira Braz Caurio	Aceito
Outros	termo_anuencia_UFCSPA.jpg	14/10/2016 10:43:07	Cássia Ferreira Braz Caurio	Aceito

Outros	TERMO_DE_COMPRIMISSO_UFCSPA.jpg	14/10/2016 10:42:39	Cássia Ferreira Braz Caurio	Aceito
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Outros	declaracao_confidencialidade.jpg	14/10/2016 10:34:05	Cássia Ferreira Braz Caurio	Aceito
Declaração de Instituição e Infraestrutura	declaracao_de_autorizacao_chefia_responsavel.jpg	14/10/2016 10:26:30	Cássia Ferreira Braz Caurio	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	declaracao_utilizacao_dados_bio.jpg	14/10/2016 10:25:37	Cássia Ferreira Braz Caurio	Aceito
Folha de Rosto	folhaderosto.pdf	14/10/2016 10:23:14	Cássia Ferreira Braz Caurio	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 16 de Novembro de 2016

Assinado por:
ELIZETE KEITEL
(Coordenador)

7.2 ANEXO II- Parecer Consubstanciado do CEP da UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Comparação entre os métodos de antigenemia e de PCR em tempo real para citomegalovírus em transplantados renais.

Pesquisador: ALESSANDRO COMARÚ PASQUALOTTO

Área Temática:

Versão: 1

CAAE: 61143916.7.3001.5345

Instituição Proponente: Irmandade da Santa Casa de Misericórdia de Porto Alegre - ISCMPA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.885.683

Apresentação do Projeto:

Resumo:

Introdução: O transplante renal é a principal metodologia para pacientes com prejuízos irreversíveis das funções renais. Atualmente, o Brasil é o

segundo em número absoluto de transplantes renais, sendo o citomegalovírus (CMV) o patógeno considerado de maior importância nesses

pacientes, visto que pode ser transmitido ao receptor pelo órgão transplantado ou por transfusões sanguíneas; a reativação do vírus latente também

pode ocorrer, bem como a reinfecção por outro sorotipo diferente. Esse microrganismo pode causar efeitos diretos e indiretos e está associado a

uma alta taxa de morbidade e mortalidade. O monitoramento para esse patógeno ocorre no pré-transplante por meio da sorologia, a fim de

classificar o receptor e o doador quanto à exposição prévia ao vírus. E, no pós-transplante, pelas técnicas de histopatologia, cultura, sorologia,

antigenemia e ensaios moleculares para detecção e quantificação de ácido nucleico para CMV (NAT). A antigenemia é a técnica adotada na Santa

Casa de Misericórdia de Porto Alegre para iniciar e monitorar o tratamento preemtivo; contudo, o

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panorama mundial indica que grande parte
laboratórios já migraram para o NAT, tendo em vista sua sensibilidade e especificidade superior. Recentemente, a Organização Mundial de Saúde
disponibilizou um Padrão de Referência Internacional, permitindo, assim, melhor padronização dos ensaios de NAT entre os laboratórios.
Internacionalmente, diversos estudos clamam pela harmonização dos padrões desse teste, o que só ocorrerá quando mais pesquisas sejam
realizadas com esse fim. Objetivo: Comparar a sensibilidade das metodologias de antigenemia e de PCR em tempo real no pós-transplante renal
para o CMV.Materiais e Métodos: Estudo de coorte prospectivo observacional unicêntrico. Serão incluídos pacientes maiores de 18 anos, indicados
para transplante renal no Hospital Dom Vicente Scherer, diagnosticados com doença renal crônica, que assinarem o termo de consentimento livre e
esclarecido (TCLE). O resultado dos exames sorológicos serão obtidos através do banco de dados, via prontuário eletrônico, uma vez que estes são
realizados na rotina pré-transplante. Uma ficha clínica será preenchida com os dados pertinentes para atender aos objetivos secundários, os dados
demográficos complementares serão pesquisados também nos bancos de dados da Instituição. No pós-transplante quatro amostras do paciente em
30, 45, 60 e 90 dias após o transplante serão analisadas, visando compara-las com os resultados obtidos da antigenemia, que também é realizada
como protocolo pós-transplante na instituição de pesquisa. Essas amostras são coletadas para os exames de rotina pós-transplante, parte delas
serão destinadas ao exame de antigenemia e o excedente ao estudo em questão.Aspectos éticos: Este projeto foi enviado para o Comitê de Ética
em Pesquisa da Irmandade da Santa Casa de Misericórdia de Porto Alegre, bem como para o Comitê de Ética em Pesquisa em Seres Humanos da
Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

Objetivo da Pesquisa:

Objetivo Primário:

• Comparar a sensibilidade das metodologias de antigenemia e de PCR em tempo real no pós-transplante renal para a detecção de CMV.

Objetivo Secundário:

• Determinar pontos de corte para o método de PCR em tempo real para CMV, em amostras de plasma, que possam diferenciar infecção de
doença; • Determinar o custo para ambos os testes e calcular custo/benefício de seus usos;• Avaliar os fatores de risco para CMV nos transplantados renais na Santa Casa;• Avaliar a soroprevalência de doadores e receptores de enxerto renal na Santa Casa no período de Janeiro a Outubro de 2017;• Avaliar a incidência de infecção ativa por CMV nos primeiros três meses pós-transplante renal, por qualquer dos métodos diagnósticos empregados;• Documentar as estratégias de prevenção e detratamento para CMV em transplantados renais na instituição;• Avaliar quais os principais patógenos relacionados a co-infecções com o CMV, nesta coorte;• Avaliar os principais efeitos indiretos do CMV em pacientes no pós-transplante renal;• Avaliar a morbidade e a mortalidade nos pacientes transplantados renais na Santa Casa, no período do estudo;•
Avaliar a prevalência de resistência aos medicamentos administrados como tratamento para CMV em pacientes transplantados renais, no período estudado.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos referentes ao estudo estão relacionados a coleta de sangue venoso periférico, podendo gerar desconforto ao paciente, bem como hematomas.

Benefícios:

A participação nesse estudo gerará benefício para os pacientes que farão esta mesma cirurgia no futuro, pois uma forma mais rápida e melhor de diagnosticar esse vírus pode diminuir os problemas causados em decorrência da doença, como, por exemplo, a rejeição do órgão.

Comentários e Considerações sobre a Pesquisa:

Trata-se de um projeto onde a UFCSPA é coparticipante.

Considerações sobre os Termos de apresentação obrigatória:

Ficaram dúvidas como: Com será a coleta? Não foi possível ler o arquivo da carta de submissão. Esclarecer o financiamento do projeto. Escrever por extenso as siglas do TCLE.
porém, trata-se de um projeto onde a UFCSPA é coparticipante.

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Conclusões ou Pendências e Lista de Inadequações:

Recomenda-se aprovar

Considerações Finais a critério do CEP:

De acordo com o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_797495.pdf	19/10/2016 08:46:16		Aceito
Outros	cartasubmissao.jpeg	19/10/2016 08:45:14	Cássia Ferreira Braz Caurio	Aceito
Cronograma	cronogramanovocep.pdf	19/10/2016 08:43:17	Cássia Ferreira Braz Caurio	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	19/10/2016 08:42:59	Cássia Ferreira Braz Caurio	Aceito
Projeto Detalhado / Brochura Investigador	projetonovo.pdf	19/10/2016 08:41:45	Cássia Ferreira Braz Caurio	Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_797495.pdf	14/10/2016 10:46:56		Aceito
Orçamento	orcamento.jpg	14/10/2016 10:44:57	Cássia Ferreira Braz Caurio	Aceito
Outros	termo_anuencia_UFCSPA.jpg	14/10/2016 10:43:07	Cássia Ferreira Braz Caurio	Aceito
Outros	TERMO_DE_COMPRIMISSO_UFCSPA.jpg	14/10/2016 10:42:39	Cássia Ferreira Braz Caurio	Aceito
Outros	formulario_inscricao.jpg	14/10/2016 10:41:57	Cássia Ferreira Braz Caurio	Aceito
Outros	declaracao_prontuarios_publicacao.jpg	14/10/2016 10:40:32	Cássia Ferreira Braz Caurio	Aceito
Outros	declaracao_isencao_onus.jpg	14/10/2016 10:38:39	Cássia Ferreira Braz Caurio	Aceito
Outros	declaracao_confidencialidade.jpg	14/10/2016 10:34:05	Cássia Ferreira Braz Caurio	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.pdf	14/10/2016 10:31:47	Cássia Ferreira Braz Caurio	Aceito
Declaração de Instituição e Infraestrutura	declaracao_de_autorizacao_chefia_responsavel.jpg	14/10/2016 10:26:30	Cássia Ferreira Braz Caurio	Aceito

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Continuação do Parecer: 1.885.683

Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	declaracao_utilizacao_dados_bio.jpg	14/10/2016 10:25:37	Cássia Ferreira Braz Caurio	Aceito
Cronograma	cronograma1.pdf	14/10/2016 10:24:50	Cássia Ferreira Braz Caurio	Aceito
Folha de Rosto	folhaderosto.pdf	14/10/2016 10:23:14	Cássia Ferreira Braz Caurio	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 05 de Janeiro de 2017

Assinado por:
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(Coordenador)

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7.3 Parecer do Comitê de Ética da UFCSPA

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