

Evaluation of diagnostic tests for cytomegalovirus active infection in renal transplant recipients

Avaliação de métodos diagnósticos para infecção ativa por citomegalovírus em receptores de transplante renal

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Submitted on: 5/29/2016.
Approved on: 12/2/2016.

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DOI: 10.5935/0101-2800.20170008

ABSTRACT

Introduction: Cytomegalovirus (CMV) infection is a main viral infection after kidney transplantation. The diagnostic methods currently employed are pp65 antigenemia and nucleic acid amplification by polymerase chain reaction (PCR) and aim at detecting viral replication. **Objective:** The goal of this study was to evaluate and compare by both methods the incidence of CMV active infection in kidney transplant patients and to establish the best clinical-laboratory correlation. **Methods:** Thirty sequential kidney transplant recipients were enrolled in a single center prospective cohort study. Peripheral blood samples were drawn from day 15 until the 6th month after transplantation and tested for CMV replication by pp65 antigenemia and quantitative PCR assays (qPCR). **Results:** Two hundred forty samples were analyzed and the incidence of active infection was similar by both methods. Time elapsed to the first positive test was almost identical but more samples tested positive by qPCR than by antigenemia in a behavior that was almost evenly distributed overtime. Agreement between tests was observed in 217 samples (90.4%; kappa = 0.529; $p < 0.001$) and in 25 patients the tests were concordant (83.3%; kappa = 0.667; $p < 0.001$). The evaluation of the diagnostic parameters for CMV replication revealed higher sensitivity for the qPCR test (82.1%) against antigenemia (59.0%). Quantitative PCR was also slightly more accurate than antigenemia. **Conclusion:** Our data demonstrate that both methods are suitable and have almost equivalent accuracy for the detection of post-transplant cytomegalovirus replication. The choice for either test must take in consideration the demand, execution capability and cost-effectiveness at each institution.

Keywords: cytomegalovirus; immuno-histochemistry; kidney transplantation; polymerase chain reaction.

RESUMO

Introdução: Citomegalovírus (CMV) é uma importante causa de infecção viral após o transplante renal. Os métodos diagnósticos presentemente utilizados são a antigenemia pp-65 e os métodos que utilizam a amplificação de ácidos nucleicos pela reação em cadeia da polimerase (PCR) e visam à detecção da replicação viral. **Objetivo:** O objetivo deste estudo foi avaliar e comparar a incidência de infecção ativa por CMV em pacientes transplantados renais pelos dois métodos e estabelecer a melhor correlação clínico-laboratorial. **Métodos:** Trinta pacientes transplantados renais seqüenciais em um único centro foram incluídos em um estudo de coorte prospectiva. Amostras de sangue periférico foram coletadas a partir do 15º dia até o 6º mês pós-transplante e avaliadas para replicação de CMV por Antigenemia pp-65 e PCR quantitativo (qPCR). **Resultados:** Foram analisadas 240 amostras e a incidência de infecção ativa foi similar pelos dois métodos. O tempo médio transcorrido desde o transplante até o primeiro teste com resultado positivo foi quase idêntico entretanto mais amostras tiveram resultado positivo por qPCR do que antigenemia, um comportamento que se manteve quase uniforme ao longo do tempo. Concordância entre os testes foi observada em 217 amostras (90,4%; kappa = 0,529; $p < 0,001$) e em 25 pacientes (83,3%; kappa = 0,667; $p < 0,001$). A avaliação dos parâmetros diagnósticos para replicação de CMV revelaram maior sensibilidade para qPCR (82,1%) contra antigenemia (59,0%). PCR quantitativo também foi levemente mais preciso do que antigenemia. **Conclusão:** Nossos dados demonstram que ambos os métodos são adequados e tem precisão quase equivalente para a detecção da replicação do CMV após o transplante renal. A escolha entre um ou outro deve levar em consideração a demanda, capacidade de execução e custo-efetividade em cada instituição.

Palavras-chave: citomegalovírus; transplante de rim; reação em cadeia da polimerase; imuno-histoquímica.

INTRODUCTION

Cytomegalovirus (CMV) belongs to the *herpesviridae* family and the infection by this pathogen has a high prevalence worldwide. Although the infection is usually harmless in immunocompetent host may be a major cause of morbidity and mortality in organ transplant recipients.¹ Active infection has been reported to occur between 40-100% of patients in different series of kidney transplant recipients.^{2,3}

CMV active infection and disease are more common in patients without prior exposure to the virus who receive organs from donors with latent infection or recipients with prior exposure that receive intense immunosuppressive therapy, especially employing T-cell depleting antibodies, such as anti-thymocyte globulin.²⁻⁵

In organ transplant populations both the effective therapy of active infection or disease and the use of pre-emptive strategies require an accurate and early diagnosis in order to obtain improved outcomes, therefore fast and accurate diagnostic methods are needed and are in current use.⁶⁻⁸

Currently in clinical practice the tests available to monitor CMV active disease are pp65 antigenemia, which detects the presence of the pp65 phosphoprotein on peripheral blood leukocytes and the detection of viral DNA by nucleic acid amplification methods, such as polymerase chain reaction (PCR), which may be either qualitative or quantitative.⁹⁻¹⁷ Serological methods to detect immunoglobulins of the IgM and IgG classes and viral cultures are not suitable for use in clinical practice due to their low accuracy and excessive time for results.¹¹⁻¹³

Previous studies demonstrated a good correlation between the quantitative PCR assays (qPCR) and pp65 antigenemia in detecting viral replication.^{6,10,18-25} It is currently recommended that both antigenemia and qPCR may be used for monitoring viral replication and response to antiviral therapy. The choice of either method basically depends on the availability of personnel and economic resources at health institutions.^{11,18,20,26,27}

The goal of this study was to evaluate the accuracy of these tests in a longitudinal study including patients that received a kidney transplant at our institution.

MATERIALS AND METHODS

PATIENTS

Two hundred forty peripheral blood samples were prospectively collected between April 2012 to February 2013 from 30 out of 100 patients submitted to kidney transplantation in our institution at this period who accepted to participate and could attend the study schedule and according to the study budget. Patients agreed to participate and were included regardless of pre-transplant CMV IgG status or induction therapy. Samples were drawn sequentially at 15, 30, 45, 60, 75, 90, 120, 150 and 180 days after transplantation. Viral replication was accessed by the pp65 antigenemia detection assay and viral DNA amplification by qPCR.

The immunosuppressive regimen consisted of combining tacrolimus, sodium mycophenolate and prednisone for all patients. Patients who received kidneys from deceased donors underwent induction therapy with Basiliximab or polyclonal T-cell depleting antibodies. In addition to the protocol samples, additional antigenemia samples were taken as needed for clinical management.

When available CMV-specific IgM and IgG serologies were obtained from the donor and recipient before transplantation. Both intravenous and oral ganciclovir doses were adjusted according to MDRD estimated GFR.

Patients at a high risk for the development of infection, CMV/IgG- recipient receiving an organ from a CMV/IgG+ donor and those receiving T-cell depleting antibodies for prophylaxis or treatment of acute rejection were treated with ganciclovir intravenously followed by oral treatment with ganciclovir up to six months after transplantation. Patients at a moderate risk, CMV/IgG+ donor and recipient or CMV/IgG+ recipient only, were monitored with serial antigenemia and treated preemptively. The diagnosis of CMV active infection was made by positivity of pp65 antigenemia assay without the knowledge of the qPCR assay. Active infection was treated with intravenous ganciclovir at doses adjusted for the graft's function.

METHODS

PP65 ANTIGENEMIA

After extraction from peripheral blood, leukocytes were incubated with monoclonal antibodies C10/C11

and others reagents of CMV Brite Turbo according to the manufacturer's recommendations (IQ® Products, Groningen, Netherlands). Leukocytes with positive antigenemia showed a homogeneous yellow-green nuclear pattern when viewed using a fluorescence microscope. The result of pp65 antigenemia was considered diagnostic for viral replication when there was one or more positive cells/200.000 analyzed.²¹

QUANTITATIVE POLYMERASE CHAIN REACTION

The quantification of nucleic acids was performed using the CMVQ - PCR Alert Kit (Nanogen Advanced Diagnostics, Torino, Italy) in DNA samples extracted from plasma collected in EDTA containing tubes, according to the instructions of the manufacturer. Five microliters of DNA were transferred to an amplification microplate containing a mixture of reagents comprising CMV-specific primers and probes as well as an internal control and the Tac polymerase enzyme.

The procedure consists of a real-time amplification reaction on a microplate with programmable temperature variation and control and an optical fluorescence detection system simultaneously to the reaction in a thermocycler. The system was standardized in the Applied Biosystems ABI PRISM 7000 devices. The PCR result was considered diagnostic for viral replication when at least 1250 viral copies/mL were detected.²¹

STATISTICAL ANALYSIS

The occurrence of active infection was verified using the antigenemia and qPCR methods. The agreement between tests was assessed using the Kappa coefficient. The Student *t* test for independent samples was used to evaluate differences between infected and uninfected patients, by both methods, for the following variables: age of donor, age of recipient, current creatinine and estimated MDRD glomerular filtration rate²⁸ and at the 6th and the 12th months after transplantation. The level of significance was set at 5% ($p < 0.05$). The analyses were performed using the statistical software SPSS, version 20.

To establish the diagnostic parameters for both assays, viral replication was considered to be present when the result of any test was positive and absent when the result of both assays was negative. The study was approved in its technical and methodological aspects by the Research Ethics Committee of Hospital

de Clínicas de Porto Alegre in accordance with Helsinki Declaration. Patients included in the study agreed to participate and signed the informed consent.

RESULTS

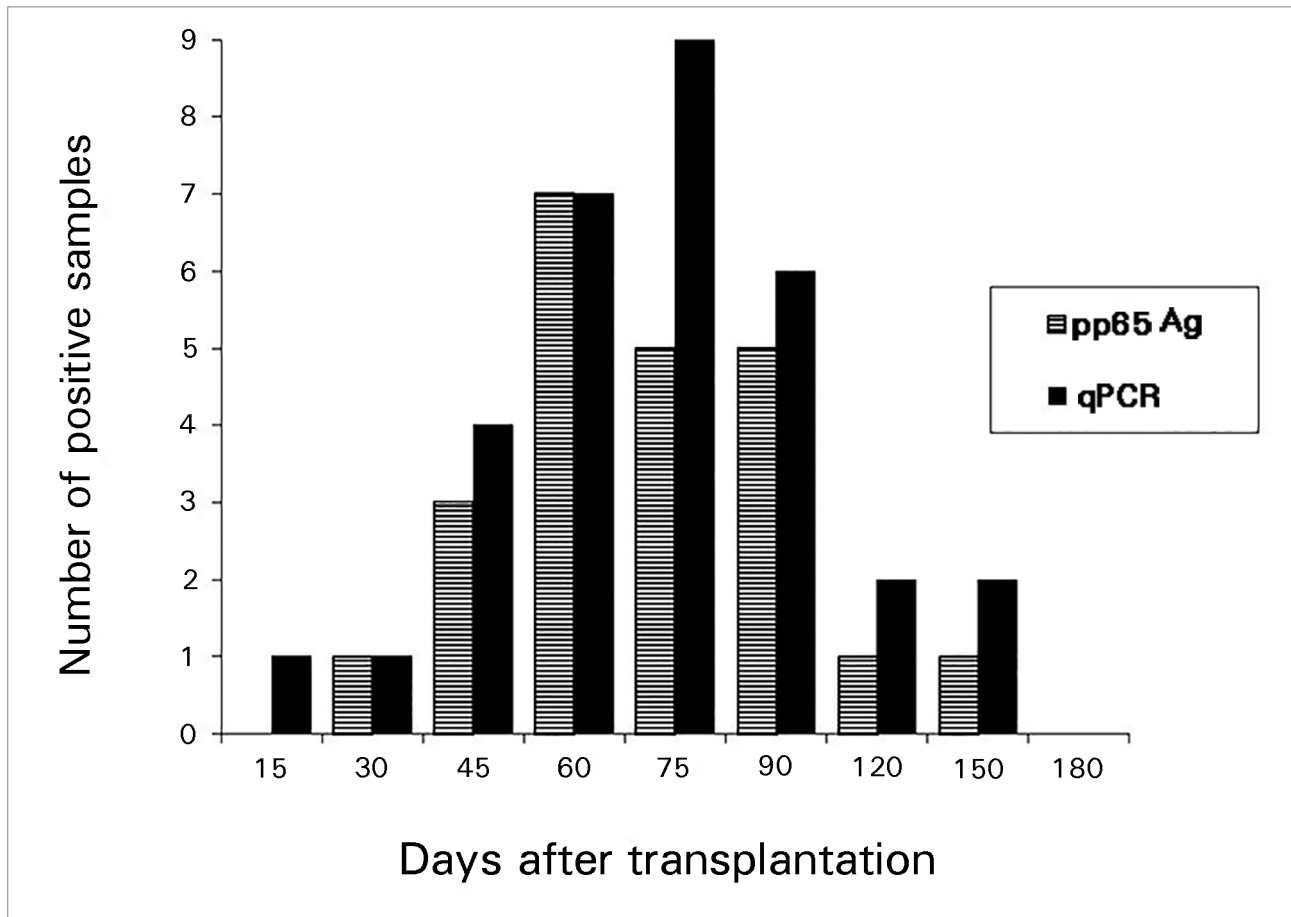
Demographic data of the studied sample are shown in Table 1. The average number of samples per patient was 8 ± 1 . In the analysis, 23 samples (9.6%) were positive by the antigenemia test and 32 (13.3%) were positive in the qPCR test. There was no difference in the mean time elapsed from the transplant to the first positive test result, which was 64 ± 23 days for antigenemia and 62 ± 21 days for qPCR ($p = 1.0$).

TABLE 1 DEMOGRAPHIC AND INITIAL IMMUNOSUPPRESSION DATA

Recipients	Frequency	%
Mean age (years, mean \pm SD)	42 ± 12	(range: 14 - 64)
White/non-white race	25/5	(83.3/16.7)
Males/females	18/12	(60/40)
CMV IgG +	30	(100)
CMV IgG -	0	(0)
Pre transplant diabetes	3	(10)
Donors		
Living/deceased	3/27	(10/90)
CMV IgG +	16	(53.3)
CMV IgG -	7	(23.3)
CMV IgG unknown	7	(23.3)
Initial Immunosuppression*		
No antibody induction	3	(10)
Induction with Basiliximab	16	(53.3)
Induction with ATG	11	(36.7)

CMV: cytomegalovirus; Induction with ATG: Induction therapy with lymphocyte-depleting antibodies. * All patients received a calcineurin inhibitor, sodium mycophenolate and prednisone.

Many samples were positive at the same time, however one sample was positive only by qPCR at 15 days after transplantation and at each time point, except for the day 30 and 60 samples, there were more positive samples by qPCR as compared with antigenemia. Also, there were no positive samples at day 180 after transplantation (Figure 1). For the samples that were positive by the antigenemia test, the mean number of positive cells were $5 \pm 6/200.000$ and for the tests that were qPCR positive the average was 5987 ± 10.623 viral copies/mL.

Figure 1. Number of positive tests by pp65 antigenemia and qPCR at each study sampling time

Due to the small number of positive samples no pattern of viral proliferation was identified for either test.

ACTIVE INFECTION DETECTED BY ANTIGENEMIA

According to this test, 16 patients (53.3%) developed active infection. In 11 recipients IgG serology was positive for both donors and recipients and in two only the recipient had antibodies. In three transplants donor serology was unknown. There was no statistically significant association between pre-transplant donor and recipient serology and the occurrence of an active infection diagnosed by this method ($p = 0.169$). No cases of cytomegalovirus disease were observed.

ACTIVE INFECTION DETECTED BY qPCR

According to this test, 15 patients (50%) developed active infection. In nine recipients IgG serology was positive for both donors and recipients, in three, only the recipient had antibodies, and IgG-CMV antibodies were present in another three in which the donor serology was unknown. There was no statistically significant association between pre-transplant donor

and recipient serology and occurrence of CMV infection diagnosed by qPCR ($p = 0.667$).

AGREEMENT BETWEEN ASSAYS AND DIAGNOSTIC PARAMETERS

Agreement between the two tests was observed in 217 samples (90.4%), both being positive in 16 (6.7%) and negative in 201 (83.7%). In seven samples (2.9%), a positive result was observed for antigenemia and a negative result for qPCR, and 16 (7.4%) tested negative by antigenemia and positive by qPCR. Among these 16 samples only 2 (12.5%) occurred in patients with low leukocyte counts (leukocytes under 4.000/ μ L).

The kappa coefficient between the antigenemia and qPCR tests was 0.529 ($p < 0.001$), which indicated moderate agreement between the tests. Over the observation period, 25 patients had concordant results for antigenemia and qPCR. Three patients tested positive by antigenemia and negative by qPCR, while two patients tested positive by qPCR and negative by antigenemia. The kappa coefficient for the observation among patients was 0.667 ($p <$

0.001), indicating a good agreement. Figure 2 shows a scatter plot of the values obtained for the two tests.

The Pearson correlation coefficient was 0.47 ($r^2 = 0.22$, $p < 0,001$), demonstrating a regular correlation between the variables. The diagnostic parameters for each assay, compared against the established gold standard, are shown in Table 2.

EFFECT OF ANTIBODY INDUCTION THERAPY AND PROPHYLAXIS

Induction therapy was used in 27 (90%) patients, 16 (53.3%) patients received anti interleukin-2 anti-receptor antibodies (Basiliximab®) and 11 (36.6%) received T-cell depleting antibodies (Thymoglobulin®). According to the antigenemia criteria active infection occurred in 13 (81.3%) of the patients who received induction therapy with Basiliximab and in only one (9.1%) of those who received induction therapy with Thymoglobulin ($p < 0.001$).

According to the qPCR criteria active infection occurred in 11 (68.8%) of 16 patients who received induction therapy with Basiliximab® and in two (18.2%) of the 11 patients receiving induction therapy with Thymoglobulin®. Three patients who received induction therapy with ATG tested positive for active cytomegalovirus infection, two by qPCR and one by

pp65 antigenemia. In all cases, the MDRD estimated GFR was higher than 60 mL/min and the ganciclovir dose administered for prophylaxis was 1 g TID.

Prophylaxis with ganciclovir was given to 15 patients. By the antigenemia criteria, three patients (20%) who received prophylactic treatment developed an active infection, while 13 (86.7%) of those who did not receive prophylactic therapy developed active infection ($p < 0.001$). In the analysis using qPCR criteria, four (26.7%) patients who were given prophylactic treatment developed active infection compared to 11 (73.3%) patients who did not receive it ($p = 0.011$).

No significant differences between patients who developed active infection and those who did not were found for donors and recipients age, gender, race, presence of *diabetes mellitus* before or after transplantation, distribution of anti-CMV serologies, antibody induction therapy or occurrence of rejection (Table 3).

MDRD estimated GFR revealed no statistically significant differences between the groups of patients with and without active CMV infection at 6, 12 months and at the last evaluation, by either diagnostic method (Figure 3). Prophylactic therapy with oral ganciclovir prevented the development of active infection under the antigenemia diagnostic criteria (RR: 0.22; 95% CI 0.07 - 0.62; $p < 0.001$) and the qPCR diagnostic criteria (RR: 0.36; 95% CI 0.15 - 0.88; $p = 0.026$).

Figure 2. Pearson's correlation coefficient between CMV pp65 antigenemia and quantitative qPCR assays. Notes: (1) To values of PCR DNA copies under 1250 copies/ml but with amplification signal it was attributed the value of 1250 copies/ml; (2) Considering the 480 tests, 88,5% of the results are equal to zero.

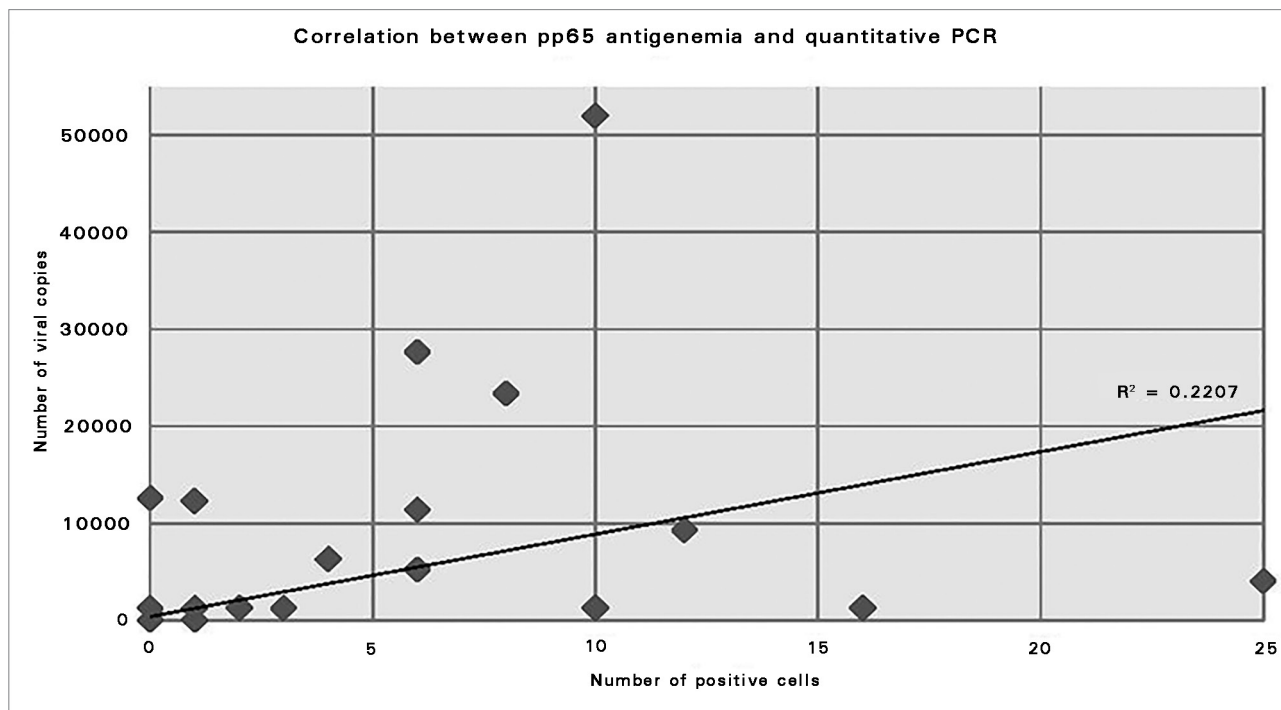


TABLE 2 DIAGNOSTIC PARAMETERS OF THE CYTOMEGALOVIRUS REPLICATION ASSAYS

Test/Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
pp65 antigenemia	59.0%	100%	100%	92.6%	93.3%
Quantitative PCR	82.1%	100%	100%	96.6%	97.1%

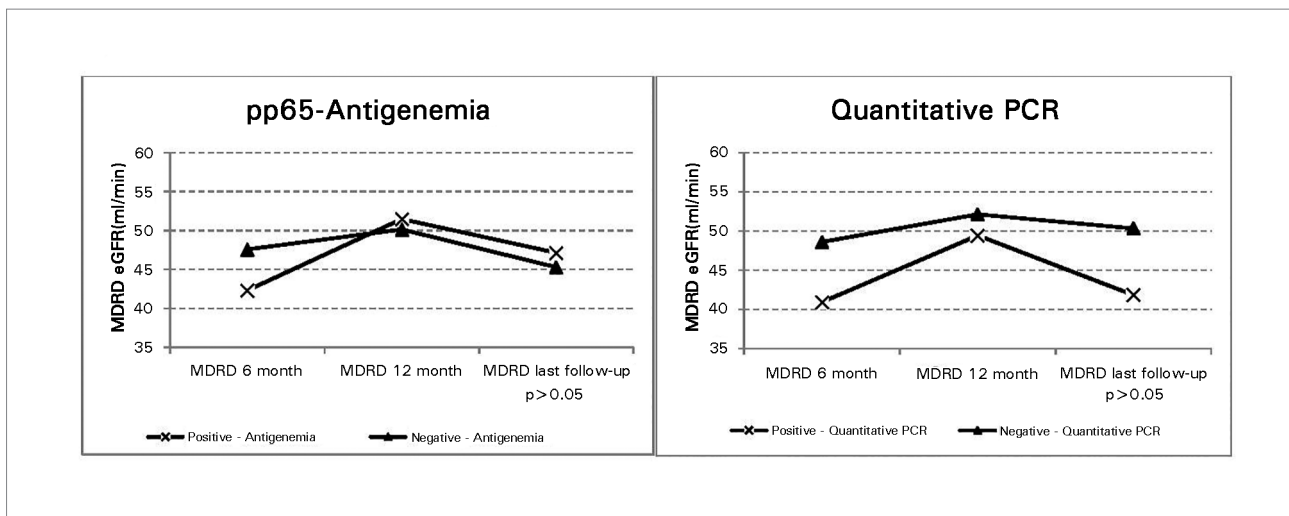
PCR: polymerase chain reaction; PPV: positive predictive value; NPV: negative predictive value.

TABLE 3 RISK FACTORS FOR THE DEVELOPMENT OF ACTIVE CYTOMEGALOVIRUS INFECTION

Test Variable	pp65 Antigenemia			Quantitative PCR		
	Infection	No infection	<i>p</i>	Infection	No infection	<i>p</i>
Age (years)	42.3	42.1	0.982	39.2	45.2	0.200
Sex (M/F)	10/6	8/6	0.765	8/7	10/5	0.456
Race (W/NW)	14/2	11/3	0.642	11/4	14/1	0.330
Pre-TxDM (Y/N)	2/14	1/13	1.0	0/15	3/12	0.224
D+R+/D-R+	11/2	5/5	0.169	9/3	7/4	0.667
Prophylaxis (Y/N)	3/13	12/2	< 0.001	4/11	11/4	0.011
Induction (Y/N)	14/2	13/1	1.0	13/2	14/1	1.0
Rejection (Y/N)	6/10	3/11	0.440	7/8	2/13	0.109

M: Male; F: Female; W: White; NW: Non-white; DM: *diabetes mellitus*

Figure 3. MDRD estimated glomerular filtration rate in patients with and without active infection evaluated by pp65 antigenemia (panel A) and qPCR (panel B). Differences are not statistically significant.



DISCUSSION

In the present study the patient’s sample is representative of the current population of transplant recipients in the southern region of Brazil. Patients are predominantly young caucasoid

males who received kidneys from deceased donors and were under antibody induction therapy. The variables involved in viral replication and related to graft outcomes were correlated to diagnostic methods and did not correlate with development of active infection.

We evaluated the two diagnostic methods currently recommended for diagnosis of active CMV infection, the pp65 antigenemia and qPCR. Both antigenemia and PCR have potential advantages and disadvantages against each other.

PCR does not require highly trained individuals, can be performed in patients with leucopenia, is automated, allows concurrent processing of multiple samples and does not require fresh biological materials. However, it requires more expensive equipment and reagents, especially in the quantitative PCR (qPCR) method, which is more accurate. The qPCR tests were standardized in 2010 by the World Health Organization (WHO), which provided a reference standard obtained from the National Institutes of Biological Standards and Controls in the UK. The standard titer is 5×10^6 IU/mL, and commercial and laboratory tests should be recalibrated so as to show collinearity with this Reference.^{8,29} Qualitative PCR, due to its low specificity, is not currently used for the diagnosis of CMV infection.^{9,11,18,30}

Pp65 antigenemia is a semi-quantitative method which detects, through immunohistochemistry or immunofluorescence techniques, the presence of the phosphoprotein 65 expressed in peripheral blood leukocytes infected with CMV.^{11-13,18,31}

Among its advantages are the high sensitivity and specificity, low cost and its easiness to perform, without need for sophisticated equipments. However, it requires trained personnel, the whole blood sample must be processed within 6 to 8 hours, and it loses sensitivity in the presence of neutropenia. Furthermore, antigenemia can be negative or with low counts in cases of tissue-invasive disease. Finally, this method is time consuming and necessitates intensive work by the laboratory staff.^{2,3,11,13}

The evaluation of the main goal of this study, to evaluate the correlation between the two diagnostic methods, showed that they have a good correlation. This is in agreement with literature data for the pp65 antigenemia and qPCR methods for the diagnosis of active CMV infection.^{12,18-20,25,32} Rhee *et al.*¹⁸ analyzed the results of pp65 antigenemia and qPCR in 899 samples taken from 111 kidney transplant patients in the recent post-transplant period, and the patients included in this study had a demographic profile similar to the profile of that population. The authors reported agreement in 84% of samples and a statistically significant correlation between the diagnostic methods.

Cariani *et al.*¹⁹ compared 475 consecutive samples obtained from 156 with transplants (kidney and bone marrow), HIV-infected and patients with hematologic malignancies, and observed a significant correlation between the tests with agreement in 77% of samples. Gouarin *et al.*²² analyzed 248 specimens from 21 kidney transplant patients and found a significant correlation between qPCR in whole blood and pp65 antigenemia considering CMV qPCR in whole blood a suitable alternative for diagnosing and monitoring CMV infection in renal transplant patients.

Piiparinen *et al.*²⁴ found an almost linear correlation between the results of the two tests in 253 consecutive blood samples from liver and kidney transplant patients. Also, Mengelle *et al.*²⁵ found a good correlation between qPCR with DNA extracted from leukocytes and pp65 antigenemia in 198 blood samples from 14 kidney, liver and heart transplant patients and considered qPCR a good alternative test to pp65 antigenemia assay.

In our study, possibly because of the gold standard criteria for diagnosis of viral replication, qPCR showed higher sensitivity for the identification of viral replication, however all other diagnostic parameters were similar. In support it is important to note that qPCR provided more positive samples than the antigenemia in most of the scheduled sampling of the study.

This study has some limitations. Firstly, the sample size is limited, and there is also a considerable percentage of donors whose IgG serology was not available for assessment, which impaired the analysis of this risk factor. In addition, the follow-up time could have been longer, and, lastly, there were no cases of tissue-invasive disease. In spite of these limitations, the results showed that the sample size was suitable for the assessment of the diagnostic methods tested.

We concluded that pp65 antigenemia and qPCR are comparable methods for the detection of viral replication. The choice of one method over the other should take into account local factors, expertise, costs and the required number of tests to be carried out in a particular transplant program depending on the transplant program strategy on monitoring CMV active infection and disease. However, qPCR is probably more sensitive and slightly more accurate test for the detection of viral replication and may replace pp65 antigenemia as the need for testing increases in transplant programs.

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