



Article/Artigo

Comparison of the performance of polymerase chain reaction and pp65 antigenemia for the detection of human cytomegalovirus in immunosuppressed patients

Comparação do desempenho da reação em cadeia da polimerase e antigenemia pp65 para detecção de citomegalovírus humano em pacientes imunossuprimidos

Patrícia Borba Martiny¹, Fernanda de-Paris¹, Alice Beatriz Mombach Pinheiro Machado¹, Ricardo Obalski de Mello¹, Martha Bergman Senger², Maria Clara Medina Corrêa², Luiz Carlos Werres Junior² and Carolina Fischinger Moura de Souza³

ABSTRACT

Introduction: Human cytomegalovirus (HCMV) is often reactive in latently infected immunosuppressed patients. Accordingly, HCMV remains one of the most common infections following solid organ and hemopoietic stem cell transplantations, resulting in significant morbidity, graft loss and occasional mortality. The early diagnosis of HCMV disease is important in immunosuppressed patients, since in these individuals, preemptive treatment is useful. The objective of this study was to compare the performance of the in-house qualitative polymerase chain reaction (PCR) and pp65 antigenemia to HCMV infection in immunosuppressed patients in the Hospital de Clínicas of Porto Alegre (HCPA). **Methods:** A total of 216 blood samples collected between August 2006 and January 2007 were investigated. **Results:** Among the samples analyzed, 81 (37.5%) were HCMV-positive by PCR, while 48 (22.2%) were positive for antigenemia. Considering antigenemia as the gold standard, sensitivity, specificity, positive predictive values and negative predictive values for PCR were 87.5%, 76.8%, 51.8% and 95.5% respectively. **Conclusions:** These results demonstrated that qualitative PCR has high sensitivity and negative predictive value (NPV). Consequently PCR is especially indicated for the initial diagnosis of HCMV infection. In the case of preemptive treatment strategy, identification of patients at high-risk for HCMV disease is fundamental and PCR can be useful tool.

Keywords: Human cytomegalovirus. Antigenemia. Polymerase chain reaction. Immunosuppressed patients. Preemptive therapy.

RESUMO

Introdução: O citomegalovírus humano (HCMV), causador de infecção latente, reativa com frequência em pacientes imunossuprimidos. Portanto, o HCMV permanece uma das infecções mais comuns após transplantes de órgãos sólidos e de células hematopoiéticas resultando em significativa morbidade, perda do enxerto e ocasional mortalidade. Assim, o diagnóstico precoce para uma terapia preventiva é de grande importância. Este estudo visa comparar o desempenho dos métodos PCR qualitativo *in-house* e antigenemia pp65 para o diagnóstico de infecção por CMV em pacientes imunossuprimidos do Hospital de Clínicas de Porto Alegre. **Métodos:** O estudo foi realizado em 216 amostras de sangue total (EDTA) coletadas de 85 pacientes, entre agosto de 2006 e janeiro de 2007. **Resultados:** Dentre as 216 amostras analisadas, 81 (37,5%) amostras apresentaram resultados positivos na PCR, enquanto 48 (22,2%) apresentaram resultados positivos na antigenemia. A sensibilidade, especificidade, valor preditivo positivo e valor preditivo negativo para a PCR, considerando antigenemia como padrão foram 87,5%, 76,8%, 51,8% e 95,5%, respectivamente. **Conclusões:** Estes resultados demonstraram que a PCR tem alta sensibilidade e valor preditivo negativo. Consequentemente PCR é especialmente indicada para o diagnóstico inicial de infecção por HCMV. No caso da estratégia de terapia preventiva, a identificação de pacientes com alto risco para a doença por HCMV é fundamental e a PCR pode ser uma ferramenta útil.

Palavras-chaves: Citomegalovírus humano. Antigenemia. Reação em cadeia da polimerase. Pacientes imunossuprimidos. Terapia preemptiva.

1. Unidade de Microbiologia e Biologia Molecular, Serviço de Patologia Clínica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS. 2. Unidade de Bioquímica e Imunoensaios, Serviço de Patologia Clínica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS. 3. Serviço de Genética, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS.

Address to: Dra. Fernanda de Paris. Unidade Microbiologia e Biologia Molecular/Serviço Patologia Clínica/HCPA. Rua Ramiro Barcelos 2350, 90035-930 Porto Alegre, RS, Brasil.

Phone: 55 51 3359-8860

e-mail: fparis@hcpa.ufrgs.br

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INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous pathogen and frequently reactive in latently infected immunosuppressed patients^{1,2}. Accordingly, HCMV remains one of the most common infections following solid organ and hemopoietic stem cell transplantations, resulting in significant morbidity, graft loss and occasional mortality. The adverse impact of HCMV infection on graft function underscores the importance of HCMV diagnosis for these patients³. In addition, nowadays a preemptive treatment approach is preferable to universal antiviral prophylactic treatment for HCMV infection⁴. Therefore, laboratory techniques are important for monitoring and diagnosing HCMV infection in immunosuppressed patients.

Serological techniques have no role in the diagnosis of active HCMV disease posttransplantation. Viral culture of blood to detect HCMV has limited clinical utility for disease diagnosis due to poor sensitivity³. Thus, diagnosis of HCMV infection includes the development of assays to determine infectious viruses (viremia), pp65-positive leukocytes (antigenemia) and viral genome in the blood (DNAemia)⁴. Antigenemia is the first laboratory method routinely used that provides a quantitative parameter for determining the proportion of the antigen pp65 in the nucleus of infected neutrophils in the blood of patients⁵. In recent years, PCR has been widely used in the detection of the HCMV genome, with greater sensitivity compared to that of viral isolation in cell cultures. PCR can provide quantitative and qualitative results with certain advantages, such as greater flexibility with samples, storage at -20°C and the possibility of repeating tests^{6,7}. The use of PCR with peripheral blood leukocytes is also more appropriate in cases of small loads, such as when antiviral therapy is already in progress⁴. In addition, a qualitative

PCR assay may be more appropriate for diagnosis of the disease than for monitoring treatment response. Qualitative PCR can be an option for surveillance if this is the only testing option available³.

However, no clinical consensus exists regarding which techniques must be applied to follow immunosuppressed patients at risk of HCMV disease. Considering this fact, this study aimed to contribute to current understanding of the advantages and disadvantages of each technique, using the STARD consensus to improve the evaluation process⁸.

METHODS

The study was conducted on 216 blood samples (EDTA) collected between August 2006 and January 2007, obtained from attendances at a tertiary hospital, Hospital de Clínicas in Porto Alegre, capital of the State of Rio Grande do Sul, Brazil. The samples were subjected to the antigenemia technique by indirect immunofluorescence (Brite™ Turbo), performed simultaneously with the qualitative polymerase chain reaction (Nested PCR). To perform the antigenemia assay, a cell fraction was separated using dextran (enriched fraction of polymorphonuclear cells). Monoclonal antibodies were used in this fraction directed against the viral antigen pp65, a protein from the HCMV matrix. The presence of antigen pp65 was detected in the nucleus of infected neutrophils by indirect immunofluorescence. The quantitative reading of 2×10^5 PBL (peripheral blood leukocytes) was used. This procedure follows the instructions provided by IQ Products®, manufacturer of the CMV Bride™ Turbo Kit®.

The samples tested by PCR were subjected to leukocyte separation by density gradient. In this technique, these cells are suspended in sterile water and the viral DNA is extracted using the QIAmp Viral® (Qiagen®) kit according to the manufacturer's instruction manual. An in-house technique of nested PCR was used for molecular determination. This technique uses two stages of consecutive amplification, with the product of the first reaction (347bp) serving as the target for the second (297bp); i.e., in this technique the primers from the second amplification are located inside the primers from the first amplification. The external primers (5' TGAGG AAT GTC AGC TTC 3' & 5' TCATGAGG TCG TCC AGA 3') and internal primers (5' CCA GCC TCA AGA TCT TCA T 3' & 5' TCG TCC AGA CCC TTGAGG TA 3') amplify fragments of the B glycoprotein gene present in the DNA of HCMV¹⁰.

Each PCR reaction in the first stage contained 16mM $(\text{NH}_4)_2\text{SO}_4$, 67mM Tris-HCl (pH 8.8 to 25°C), 1.5mM MgCl_2 , 0.01% (w/v) of Tween-20, 250mM dNTP, 5µM of external primers and 1.25U of polymerase DNA Super-Therm (JMRHolding®). The final volume of the reaction mixture was 50µL, containing 10µL of DNA

extracted from the sample. Amplification was conducted in a Techne® thermocycler. The reaction began with denaturing at 94°C for 1min and 40s, followed by 33 cycles at 94°C for 30s, annealing at 50°C for 30s and polymerization at 72°C for 30s. In the second amplification reaction, a reaction mixture identical to the first was used; however, with half of the individual volumes of the reagents and using the internal instead of the external primers and the addition of 2µL of the amplicon obtained in the first reaction. The reaction began with denaturing at 94°C for 45 minutes and 45 seconds, followed by 33 cycles at 94°C for 20s, annealing at 50°C for 30s and polymerization at 72°C.

The final products of these reactions were electrophoresed on 2% agarose gels stained with 0.5µg/mL ethidium bromide. All testing was performed with negative and positive controls. As negative control, a reaction containing 10µL of sterile water was used instead of the clinical sample. As positive control, an aliquot of NAT (nucleic acid total) of HCMV (Virion®) was amplified. The results were expressed qualitatively as positive or negative.

For the statistical analysis, the Pearson Chi square test with Yate's correction was used to compare the number of positive samples in both techniques. The level of significance used was $p < 0.05$. The agreement between both techniques was determined by the Kappa coefficient. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the PCR technique using antigenemia as the gold standard.

Ethical considerations

The protocol study was approved by the hospital's research ethics committee.

RESULTS

Of the 216 samples collected in the period described, 85 patients were studied, of which 41 were kidney transplant patients, 24 were bone marrow recipients, 10 were receiving chronic use of immunosuppressive drugs, 8 were liver transplant patients and 2 were HIV seropositive individuals. The positivity rates obtained by both techniques for the 216 samples tested are presented in (Table 1).

Among the 81 samples positive by PCR, 39 (48.1%) samples were negative in the antigenemia testing. On the other hand, among the 135 samples negative by PCR, only 6 (4.4%) showed a positive result for antigenemia (Figure 1).

Considering antigenemia as the gold standard, sensitivity, specificity, positive predictive values and negative predictive values for PCR were 87.5%, 76.8%, 51.8% and 95.5%, respectively.

The Kappa value obtained for agreement between both techniques was 0.516.

TABLE 1 - Percentage positivity of human cytomegalovirus using polymerase chain reaction and antigenemia.

	Positive samples		Negative samples		Total	
	n	%	n	%	n	%
Polymerase chain reaction	81	37.5*	135	62.5	216	100.0
Antigenemia	48	22.2	168	77.8	216	100.0

*statistical significance. $\chi^2_{2,0.05,1} = 11.318, p = 0.001$ by the Pearson chi-square test with Yate's correction, PCR: polymerase chain reaction.

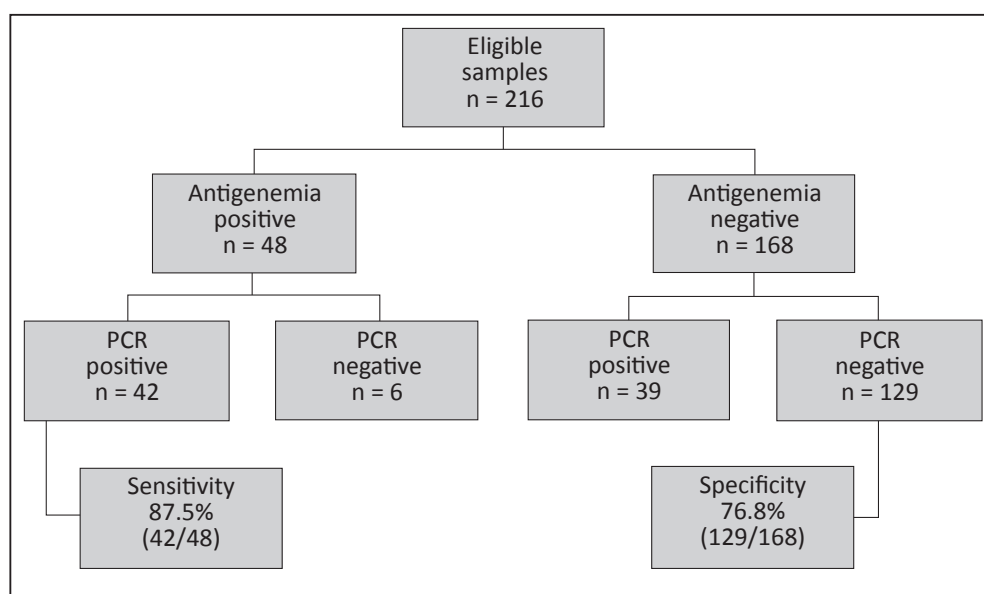


FIGURE 1 - Flowchart showing the results for human cytomegalovirus screening by antigenemia (kit Brite™ Turbo) vs PCR (in-house).

DISCUSSION

Human cytomegalovirus infection is a severe pathology and widely affects transplant patients^{4, 5, 11-13}. The concern with the worsening of a patient's health by cytomegalic disease is clearly revealed in the present sample, since of the total of 85 patients followed-up for HCMV viremia, 73 were transplant patients: 41 kidney, 24 bone marrow and 8 liver transplant patients. Analysis of the data showed that the PCR technique was capable of detecting a higher number of positive samples (81/216) compared to antigenemia (48/216), a statistically significant difference. These results are in agreement with Solano et al¹⁴, who reported a greater positivity index for HCMV by PCR in plasma than by pp65 antigenemia¹⁴. Due to the use of a preemptive treatment strategy for HCMV in transplanted patients, there is a great demand for diagnostic methods that can determine viral replication prior to the manifestation of clinical symptoms. For this reason, diagnostic tests that have high sensitivity and NPV are fundamental for transplanted patients under immunosuppression¹². In this study, the sensitivity and specificity of PCR were 87.5% and 76.8%, considering antigenemia as the gold standard. Positive and negative predictive values of PCR were estimated as 51.8% and 95.5%. Solano et al¹⁴ and Ksouri et al¹⁵ reported 86.5% and 64% for PCR sensitivity. Therefore, the sensitivity value determined in this work was high and similar to that reported by Solano et al¹⁴. However, even antigenemia, which is considered the gold standard, can generate false negative results¹⁶⁻¹⁹. Thus, the supposedly false positive results in PCR could actually indicate the presence of HCMV in the samples studied. The gold-standard performance affects specificity and positive predictive value parameters. Furthermore, the comparison of the experimental results for both techniques indicated that they are not fully overlapping methodologies. The Kappa value obtained for agreement between both techniques was 0.516, which reveals a moderate agreement²⁰.

On the other hand, when analyzing the raw data from the techniques, observation revealed that among the 81 samples positive by PCR, 39 (48.1%) samples were negative by the antigenemia test. Furthermore, among the 135 samples negative by PCR, only

6 (4.4%) showed a positive result by antigenemia (Figure 1). These results attest to the importance of PCR in determining negative patients⁵. The high NPV (95.5%) showed that PCR could be used on patients without HCMV disease symptoms to indicate which patients required sequential monitoring for HCMV. Thus, patients with no symptoms of HCMV disease and negative PCR would not require monitoring by antigenemia.

The current challenge in HCMV follow-up of immunosuppressed patients is to define the most adequate diagnostic technique for a given clinical situation. Diagnosis and treatment costs and potential side effects should also be considered²¹. Viral load determination by quantitative PCR (real-time PCR) has been reported in literature as a useful technique for HCMV monitoring in immunosuppressed patients^{17,19,22}. However, many transplant centers do not have laboratories capable of quantifying HCMV in patient samples. We believe, however, that both qualitative PCR and antigenemia may be used, particularly in transplant centers that do not have access to real-time PCR. This work showed that qualitative PCR has high sensitivity and NPV. Consequently PCR is specifically indicated for the initial diagnosis of HCMV infection. In the case of preemptive treatment strategy, identification of patients at high-risk of HCMV disease is fundamental^{3,5,11,12} and PCR can be a useful tool in these cases.

Molecular monitoring by direct viral genome detection is of greatest utility for patients at high risk of HCMV infection. In this study, higher positivity rates using the PCR method for HCMV DNA screening of peripheral blood of immunosuppressed patients were obtained. Thus, this test could determine the onset of preemptive treatment and meticulous follow-up for such patients.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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