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Short Communication

Standardization of Antigenemia and qPCR Cut-off Values in Whole Blood for the Detection of Cytomegalovirus Disease in HIV Patients

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Abstract

Introduction: We defined the cut-off values of the antigenemia and cytomegalovirus (CMV) DNA tests in HIV/AIDS patients to identify CMV disease. **Methods:** A total of 97 samples from 68 patients with and without CMV disease were analyzed by viral DNA detection and antigenemia assay. **Results:** Qualitative and quantitative results significantly differed between assays. The cut-off values for the antigenemia and qPCR assays were 1.5 positive cells/200,000 leukocytes and 3.715 log/mL, respectively. **Conclusions:** Antigenemia and qPCR are suitable for monitoring CMV disease in HIV patients, however, the threshold values should be determined within the centers where the patients are monitored.

Keywords: AIDS. Cytomegalovirus. Real-time PCR. pp65 antigenemia.

Severe cytomegalovirus (CMV) disease has been reported in patients with AIDS, usually when the CD4+ T cell count is <100 cells/μL[1,2]. In such individuals, CMV reactivation can cause direct pathogenicity, leading to diseases such as retinitis, encephalitis, pneumonitis, and those involving the gastrointestinal tract[1], thus impairing target organs, causing AIDS progression, and high mortality[3]. Moreover, asymptomatic CMV infection has been associated with immunosenescence and aging in HIV-infected individuals[4].

Laboratory diagnosis of CMV infection should be performed by detecting the pp65 viral antigen or by quantifying viral DNA. The most commonly used technique is antigenemia, which is a semi-quantitative method that detects pp65 protein in the cytoplasm of peripheral blood polymorph nuclear leukocytes (PMNLs) by indirect immunofluorescence (IFI). This test depends on the technical ability to visualize fluorescent staining, is influenced by leukopenia, and has a long turnaround time[5]. Quantitative PCR is quick, highly sensitive and specific. The presence of CMV high viral loads in HIV+ patients is associated with disease. Currently, a universal cut-off value for infection or CMV-active disease has not yet been established for either test in the context of AIDS patients. Likewise, contrasting the case of transplanted patients, there is still no consensus on the benefit of the systematic monitoring of CMV replication in AIDS patients, or on the impact of pre-emptive therapy[6].

The detection of CMV replication in HIV+ individuals and the determination of a cut-off value for quantitative tests is critical to determine the impact of this disease and to establish the need for preemptive treatment to increase the survival of HIV/AIDS patients. Here, we evaluated the performance of antigenemia and real-time PCR for CMV detection in samples from patients with HIV/AIDS, admitted in a tertiary hospital.

This prospective cohort study evaluated the association of laboratory findings with clinical data to define the presence or absence of disease caused by CMV in HIV/AIDS patients. HIV-infected patients with suspected CMV infection admitted at Hospital de Clinicas, Universidade Federal do Paraná, Southern Brazil, for one year, were included in the study. Each episode of CMV disease was recorded, and a patient could have been admitted more than once during the study period. CMV disease

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was defined by the presence of CMV in the target organ and/or clinical response to treatment with ganciclovir. The Ethics Committee at Hospital de Clínicas – UFPR approved this study (IRB: #07283012.6.0000.0096).

The pp65 viral antigen assay was performed in PMNLs by an IFI assay using the CMV Brite™ Turbo kit (IQ Products, Groningen, Netherlands). Cells with fluorescent nuclei were counted as positive cells. Results were expressed as the number of positive cells in 200,000 leukocytes. Patient samples were considered positive when they had one or more cells with fluorescent nuclei.

The nucleic acids were extracted from 200 µL of whole blood using the High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) according to the manufacturer's protocols. Viral DNA was detected and quantified using the CMV Q-PCR Alert kit (Nanogen Advanced Diagnostics S.P.A., Lombardy, Italy) according to the manufacturer's instructions. The gene encoding the major immediate early antigen (MIEA) of CMV was amplified, and the gene encoding human beta-globin was used as extraction control.

Descriptive and analytical statistical analyses of data were performed, baseline demographic and clinical characteristics were presented with medians and interquartile ranges (IQRs), and differences between the groups were analyzed using Fisher's exact test or Mann–Whitney's test, as appropriate. The level of significance was set at 0.05.

The qualitative agreements between both assays were calculated using the kappa concordance index. Correlation analysis between viral load and pp65 results was performed using Pearson's coefficient analysis. Sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated for the qualitative data, and their combined results were used to define the gold standard for the presence of disease (i.e., a true positive was a specimen that was positive by two or more methods; or presence of viral inclusion suggesting CMV infection in biopsies and/or response to ganciclovir treatment). The receiver-operating characteristic (ROC) curve was adjusted to determine the cut-off point for antigenemia and qPCR for CMV.

We evaluated 97 samples collected from 68 patients (mean, 1.4 samples/patient), with a mean age of 37.77 ± 13 years and a median HIV diagnosis time of 1,539 days (IQR, 47–5,548 days), i.e., 4.2 years (IQR, 0–15.2 years). The median CD4+ T lymphocyte count was 95 cells/mm³ (IQR, 36–226 cells/mm³), and the mean HIV viral load was 4.16 ± 1.76 log/mL at the time of hospitalization. The average length of hospital stay was 16.6 ± 13.9 days.

Patients were divided into two groups: with or without CMV disease at the moment of blood collection. Thirteen (13.4%) samples were collected from patients with CMV disease, and 84 (86.6%) samples from non-CMV patients. Overall, patients who had CMV disease presented with a significantly lower CD4+ T cell count, time of HIV diagnosis, duration of antiretroviral use, and higher HIV viral load (**Table 1**).

Of the 97 samples collected, 26 (26.8%) were positive for the antigenemia assay, and 45 (46.3%) were detected by qPCR. Among the 26 samples positive for antigenemia, 25 (96.1%) were detected by qPCR, and one (3.9%) was undetected. From the 71 samples negative for antigenemia, 20 (28.1%) were detected by qPCR, and 51 (71.9%) were undetected.

The qualitative and quantitative comparison of the results between patients with and without CMV disease differed significantly for the results obtained by both antigenemia and qPCR (**Table 2**).

Table 3 shows the operational characteristics of antigenemia and qPCR. Both tests had low PPV, which depends on

TABLE 1: Epidemiological and laboratory characteristics presented by the HIV patients at the time of sample collection.

Features	Patients samples without CMV disease N = 84 (86,6%)	Patients samples with CMV disease N = 13 (13,4%)	*P* value
Current CD4+ T-cell (cells/mm³) **Median ±IQR**	90 (30-201)	17 (16-207)	**0.047**
Current HIV viral load (log/mL) **Mean ±SD**	4,01 (1,82)	5,15 (0,77)	**0.027**
Time until HIV diagnosis (days) **Median ±IQR**	1337 (49,5-5362)	47 (15,5-1789)	**0.010**
Time until HIV diagnosis and onset of CMV (days) **Median ±IQR**	NA	47 (9-1509)	NA
Time to use ART before hospitalization (days) **Median ±IQR**	135 (46-1705)	24 (14,5-26)	**0.002**

IQR: interquartile range; **ART:** antiretroviral therapy; **NA:** not applicable. **Note:** In bold – significant values.

disease prevalence. However, even with higher CMV disease prevalence, the PPV remained around 30%.

In terms of agreement for qualitative results, both results differed significantly; Cohen's kappa index (κ) was 0.552 (95%CI = 0.377–0.707), showing a moderate concordance. Quantitative comparison of antigenemia and qPCR for CMV was performed using Pearson's coefficient analysis, and a moderate correlation was found (r = 0.45, 95%CI = 0.27–0.6, p<0.0001).

Considering the defined criteria, the results of antigenemia and qPCR assays were compared between patients with and without CMV disease, and the ROC curve was adjusted. The cut-off of the antigenemia assay was 1.5 positive cells/200,000 leukocytes. The sensitivity presented at this cut-off point was 76.9% (95%CI, 54–99.8%) and specificity, 86.9% (95%CI, 79.7–94.1%) (**Figure 1**).

For the construction of the ROC curve of qPCR, only samples with viral detection were included, totaling 45 samples, of which 13 (28.9%) were collected from patients with CMV disease and 32 (71.1%) from patients without disease. The cut-off point was 3.7 log/mL. The sensitivity was 76.9% (95%CI, 54–99.8%) and specificity, 68.8% (95%CI, 52.7–84.8%).

CMV infection has a significant impact in patients with AIDS, and the use of methods to identify patients at risk of CMV disease remains a challenge in clinical practice[4,7-9]. Antigen and DNA screening tests have been considered valuable for identifying such patients and for determining preemptive therapy. Although the benefits of this treatment have already been described in transplant patients, it is not usually recommended for patients with HIV owing to its high cost, concerns about the benefits, the risk of developing resistance, and the lack of evidence of increased survival[6,10].

However, some reports have shown that the use of valganciclovir in HIV+ patients is not associated with the reduction in T-cell hyper activation caused by CMV replication, which can lead to immunosenescence and incomplete immune

TABLE 2: Results of antigenemia and qPCR between patients with or without CMV disease.

Category	Without CMV disease n=84 (%)	With CMV disease n=13 (%)	*P* value
pp65 Antigenemia			**<0.0001**
Positive	16 (19,1)	10 (76,9)	
Negative	68 (80,9)	3 (23,1)	
qPCR CMV			**<0.0001**
Detected	32 (38,1)	13 (100)	
Not detected	52 (61,9)	0 (0)	
pp65 Antigenemia positive cells/200.000 Mean (SD) Median ±IQR	2,3 (7,5) 0 (0)	55 (117) 12 (1-66)	**<0.0001**
qPCR CMV log/mL Mean (SD)	1,1 (1,6)	3,9 (0,8)	**<0.0001**

SD: standard deviation; **IQR:** interquartile range.

TABLE 3: Evaluation of the performance of antigenemia tests and qPCR for CMV detection.

	Antigenemia (CI 95%)	qPCR (CI 95%)
Sensitivity	77% (45–93%)	100% (71–100%)
Specificity	80% (70–88%)	61,9% (50–72%)
Positive predictive value (PPV)	38% (20–59%)	28% (16–44%)
Negative predictive value (NPV)	95% (87–98%)	100% (91–100%)
Likelihood Ratio (LR+)	4 (2.3–6.8)	2.62 (1.99–3.44)
Likelihood Ratio (LR-)	0.28 (0.1–0.77)	0
Prevalence (PPV)		
2%	7%	5%
5%	17%	12%
10%	30%	22%
15%	41%	31%
Prevalence (NPV)		
2%	99%	100%
5%	98%	
10%	96%	
15%	95%	

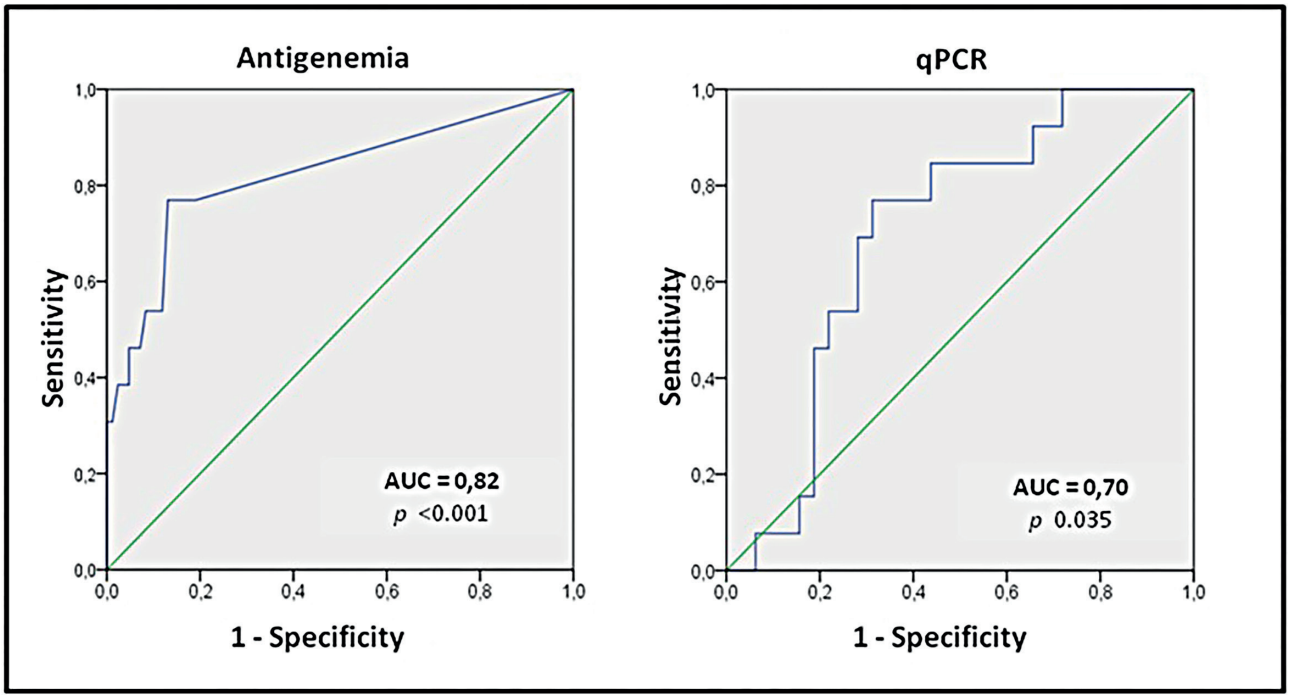

FIGURE 1: ROC curve of quantitative results of pp65 antigenemia **(A)** and qPCR **(B)** for HCMV. Note: AUC = Area under the ROC curve. *p* value for comparison of AUC between groups (with/without disease).

recovery. Thus, the use of antivirals might help improve the immune status, since the evidence of CMV replication even without disease has been reported to aggravate and increase the activation of the persistent immune response, inducing immunosenescence and accelerated aging in HIV-infected individuals[4,8,11,12,13].

Laboratory methods for the quantitative detection of CMV have been widely used for monitoring viral reactivation in immunosuppressed patients, especially those undergoing transplants[1]. However, in the context of HIV/AIDS patients, there is no consensus on the applicability in detecting active CMV infection, or of the cut-off values that allow inferring the presence of disease[6].

All individuals evaluated presented with advanced immunodeficiency and serious opportunistic infections. Most of them had CD4+ T lymphocyte counts below 50 cells/mm^3, which is an important risk factor for the onset of CMV disease, as around 40% of HIV patients with <50 cells/mm^3 might develop CMV disease[12].

As a consequence of the greater sensitivity, the implementation of molecular tests on routine diagnosis has contributed to the increase in pathogen identification in different clinical samples[5]. In a retrospective study, Beadsworth *et al*. evaluated postmortem examinations and found that CMV infection could not be detected in six of nine patients, perhaps owing to the unavailability of molecular techniques for virus detection[14]. In this study, 100% and 77% of the samples from patients with CMV disease had positive results in DNA and pp65 tests, respectively. Although the frequencies of positive and negative tests differed significantly, CMV detection has frequently not been related to active disease, and around 38% and 20% of samples from patients without CMV disease yielded positive results in DNA and pp65 tests, respectively. Therefore, quantitative evaluation is crucial in this investigation[9,12].

Similarly, a significant difference was observed in the values of quantitative results obtained by both tests in patients with and without CMV disease, making them suitable to identify HIV patients who are at a high risk of developing CMV disease. Despite the greater sensitivity observed for molecular tests, pp65 detection presented a higher PPV and likelihood ratio, and it might therefore be a valuable tool to investigate CMV disease in this cohort of patients, as its lower cost, use of equipment that is commonly available in clinical laboratories, and the need for less technical skill are important factors to be considered[9].

PPVs and NPVs differed according to the prevalence of the disease in the evaluated population; hence, we evaluated test performance considering different CMV prevalence rates, from about 2% to 15%. The antigenemia PPV values were higher than qPCR PPVs.

The ROC curve was set to find cut-off points for the variable that is associated with the outcome (of disease in this case) for both tests, and based on the curve area (0.82); antigenemia was verified to be a reliable test for the determination of CMV disease in patients with HIV. The cut-off point presented was 1.5 cells/200,000 leukocytes, and values above this point are predictors of CMV disease, indicating that patients needed preventive therapy. The cut-off value obtained from molecular test results suggests that values above 3,715 log/mL (around 5,000 copies/mL) indicate CMV disease in HIV patients.

Viral loads of 1,000 copies/mL (3 log/mL) might be appropriate for initiating preemptive treatment in HIV/AIDS

patients in ICUs, and in patients with CMV viral loads of 5,000 copies/mL (3.69 log/mL). The use of pre-emptive therapy helps prevent the onset of target organ disease in individuals with advanced HIV immunosuppression[10,15]. The differences in cut-off values obtained might be due to the distinct qPCR methods used (e.g., homemade or commercial kits), standard curve employed, and so on.

Currently, there is no gold standard for the diagnosis of active CMV infection in HIV/AIDS patients. Although antigenemia is a popular test in the monitoring of CMV, it is limited by lower sensitivity in leukopenic patients, and need of technical skills to detect positive cells. Meanwhile, qPCR, despite having high sensitivity, is not a good predictor of disease, requiring standardization and determination of the different inter-laboratory cut-off values. Therefore, there is a consensus that both techniques are suitable for CMV infection monitoring, but the method and its cut-off value should be determined within the centers where the patients are monitored.

In conclusion, we demonstrated that both pp65 antigenemia and qPCR are useful for the identification of patients at risk for CMV illness development over time, and determining the cut off values to detect CMV disease for both tests. The use of quantitative results together with the two tests in parallel can more accurately identify CMV disease in hospitalized HIV/AIDS patients. This will facilitate the development of specific therapy for human CMV and determine the relevance of preemptive therapy in such patients.

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Conflict of Interest

The authors declare there are no conflicts of interest.

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