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DIAGNOSIS OF CYTOMEGALOVIRUS INFECTIONS BY QUALITATIVE AND QUANTITATIVE PCR IN HIV INFECTED PATIENTS

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SUMMARY

A high incidence of cytomegalovirus (CMV) infections is observed in Brazil. These viruses are causatives of significant morbidity and mortality among patients with advanced human immunodeficiency virus (HIV) infection. This work, shows the application of a PCR on determination of CMV load in the buffy coat and plasma. We analyzed the samples of 247 HIV infected patients in order to diagnose CMV infection and disease. We developed a semi-quantitative PCR that amplifies part of the glycoprotein B (gB) gene of CMV. The semi-quantitative PCR was carried out only in positive clinical samples in a qualitative PCR confirmed by a nested-PCR. CD_4 lymphocyte count, HIV viral load and CMV disease symptom were correlated with CMV load. CMV genome was detected in the buffy coat of 82 of 237 (34.6%) patients, in 10 of these the CMV load was determined varying between 928 and 332 880 viral copies/μg DNA. None of these 237 patients developed any suggestive manifestation of CMV disease. For the other 10 HIV infected patients selected based on the suspicion of CMV disease, CMV genome was detected in only one case. This patient presented a high CMV load, 8 000 000 copies/μg DNA, and developed a disseminated form of CMV disease including hepatitis and retinitis. Our results were greatly influenced by the impact of the highly active antiretroviral therapy that reduced incidence of CMV viremia and occurrence of CMV disease in the HIV infected patients.

KEYWORDS: CMV; Semiquantitative PCR; HIV patients.

INTRODUCTION

Cytomegalovirus (CMV) is a genus of viruses belonging to the Herpesviridae family. These viruses have sizes that range from 150 to 200 nm, possess an envelop, a matrix and a nucleocapsid containing a double strand 240 kilobases DNA that codifies 35 structural proteins. The viral envelop contains at least 8 glycoproteins including the glycoprotein B, considered as a viral receptor and an important viral immunoresponse inducer[3]. As other viruses of this family, CMV is able of establishing host latency after a period of acute infection[16]. The exact sites that allow latent infection are unclear, but polymorphonuclear cells, dentritic cells, endothelial vascular tissue and salivary glands may contain the virus[11,12].

CMV infections are ubiquitous in Brazil and about 95% of the adult individuals are CMV seropositives[18]. After CMV infection in immunocompetent individuals, a disease occurs in a small number of cases and it is usually manifested as an infectious mononucleosis-like syndrome. It is different from what happens with immunodeficient patients where different clinical manifestations are associated to CMV disease, such as retinitis, colitis and encephalitis. These clinical manifestations occurred in 25-40% of AIDS patients before the appearing of the highly active anti-retroviral therapy (HAART)[8,13]. In these patients the disease frequently occurred as a reactivation of latent virus in CMV seropositive patients. Likewise it has been shown that CD_4 lymphocyte levels below 50 cells/mm^3 are important markers in the prognostic of clinical manifestations of CMV and as well as indicate a disease phase frequently defined as advanced AIDS[5,9].

The diagnosis of CMV infections is commonly done from blood, urine, bronchoalveolar lavage, and biopsy tissues by virus isolation in human fibroblast cell culture, detection of virus antigens in blood leukocytes by enzyme immunoassays, detection of anti-CMV antibodies by ELISA and by histopathology detecting cells containing inclusion bodies[2].

Presently, highly sensitive methods such as PCR are used in order to detect CMV in HIV infected patients. However, due to the high infection prevalence, it is difficult to interpret results of sensitive PCR tests for CMV genome detection in clinical samples because a positive result does not always mean that the virus is the effective etiological agent. In HIV infected patients the diagnosis of active infection is based on the detection of the CMV replication in the blood, it is related to high viremias as well as to a great risk to develop CMV disease. High CMV

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loads in the blood are important predictive factors for active infection and for the appearing of disease in HIV infected patients[15]. Thus quantitative PCR method for determination of CMV loads could be important tool for diagnosis of active infection and disease, as well as for CMV disease prediction in AIDS patients. In the present work we show the application of a qualitative and a quantitative PCR methods on the diagnosis of CMV infections in HIV infected patients.

MATERIAL AND METHODS

Patients: Two hundred forty seven HIV infected patients managed at the Division of Infectious Disease of the General Hospital of the School of Medicine of Ribeirão Preto, São Paulo University were included in this study. The patients, in distinct stages of infection (Centers for Disease Control and Prevention, 1993), were those studied between February 1999 to December 2000. The patients were admitted after consenting agreement. Clinical and laboratory data were obtained from the patients records, as well as use of highly active anti-retroviral therapy (HAART). The patients were separated in 2 groups: 237 patients randomly selected and 10 patients presenting clinical manifestations suggestive of CMV disease. None of the 247 patients received CMV prophylaxis or CMV antiviral treatment previously to the beginning of the study.

This research project was approved by the Ethic Board of the General Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo (Process 826/99).

An association between disease and CMV load was searched in the participating patients. Clinical manifestations such as retinitis (retin exsudate, perivascular hemorrhage), gastrointestinal involvement (colitis and esofagitis confirmed with histological findings or detection of CMV DNA *in situ*), and encephalitis (diagnosed based on the detection of CMV DNA by PCR of samples from cerebro spinal fluid) were associated to CMV disease[8].

DNA purification: Five ml of whole blood samples were collected in EDTA containing tubes. Peripheral blood leukocytes (PBL) were separated from the blood samples by centrifugation in a dextran solution (Sigma, Germany). The pellet containing the PBLs was suspended in 200 µl PBS and the DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Germany), according to the manufacturer recommendations. CMV DNA was also obtained from the supernatant of fibroblast cell cultures infected with CMV AD169 strain using the QIAamp DNA Blood Kit (Qiagen, Germany).

Qualitative PCR and nested-PCR for CMV: The reaction mixture of the qualitative PCR contained in a total volume of 50 µl, 75 mM of Tris-HCl (pH 9), 2 mM of $MgCl_2$, 50 mM of KCl, 20 mM of $(NH_4)_2SO_4$, 50 µM of each one of the deoxynucleoside triphosphates, 0.3 µM of primers gB1 and gB2[6], and 1 µg of DNA obtained from PBLs. The reaction mixture was first incubated at 94 °C for 3 min, the temperature was then reduced to 80 °C, and 2 U of Taq DNA polymerase were added. The PCR mixture was subjected to 15 cycles of 60 sec at 94 °C, 120 sec at 65 °C, and 120 sec at 72 °C, and to 30 cycles of 60 sec at 94 °C, 90 sec at 55 °C, 120 sec at 72 °C, and finally to 3 min at 72 °C. Two microliters of this reaction were used in a nested-PCR containing the same components as mentioned above, except for the internal primers gBn1 and gBn2[1]. The reaction mixture was first incubated at 94 °C for 3 min, the temperature was then reduced to 80 °C, and 1 U of Taq DNA polymerase was added. The PCR mixture was subjected to 30 cycles of 60 sec at 94 °C, 60 sec at 55 °C, and 60 sec at 72 °C, and finally to 3 min at 72 °C. PCR and nested-PCR products were subjected to electrophoresis in 2% agarose gel and the amplicons bands were visualized by UV after ethidium bromide staining.

Each PCR assay included a positive control with CMV AD169 DNA and a negative control containing distilled water. PCR for β-globin gene detection was performed in order to confirm the DNA extracts integrity. Primer nucleotide sequences are shown in Table 1.

Table 1
Primers used in the CMV qualitative, CMV semi-quantitative PCR and in the nested-PCR

Primers	Nucleotide sequence	Genome annealing site
gB1	5'GAAACGCGCGGCAATCGG 3'	81874 to 81891
gB2	5' TGGAACTGGAACGTTTGGC3'	82158 to 82176
GBn1	5'GCGCCGTTGATCCACACACC 3'	81960 to 81979
GBn2	5'TACGCTGCAGTTCACCCCAG 3'	82055 to 82055

Cloning a fragment of the gB gene of CMV into a pCR II plasmid: The amplicon of 296 bp obtained from the CMV AD169 DNA in the PCR using gB1 and gB2 primers was ligated into the plasmid vector pCR II (Invitrogen, USA). The plasmids were amplified in bacteria (*E. coli)* and purified with the Plasmid Míni Kit (Qiagen, Germany). The plasmids were sequenced with the Thermo Sequenase CY5.5 terminator kit (Amershan, England), using the M13/Forward-Reverse primers. The sequencing reaction was resolved in an automated sequencer (Seq 4 x 4, Pharmacia, USA). The obtained sequence was compared with that of the CMV AD169 strain (Genbank, sequence M60931) using the DNAsis software (Hitachi, Japan).

Plasmid quantification: The molecular weight of the plasmid 1204830 added to the insert of 296 bp molecular weight was calculated considering as 660 the average molecular weight of a base pair and was found to be 1400190. Therefore 1400190 g of the plasmid contain 6.02 x 10^{23} plasmid molecules (Avogrado's number). Thus, the number of plasmid particles in our stock solution was determined based on the plasmid DNA concentration obtained by spectrophotometric measurement at 260 nm. Aliquots of the quantified plasmid were stored at – 20 °C until use for the CMV semi-quantitative PCR tests.

Sensitivity of the CMV semi-quantitative PCR: The sensitivity of the CMV semi-quantitative PCR using primers gB1 and gB2 was determined by testing in quadruplicate decimal dilutions of the cloned plasmid solution. Amplicons were detected until the 10^{-8} dilution which corresponds to 800 plasmids.

CMV semiquantitative PCR: CMV load was determined in all the clinical samples with positive result in the qualitative PCR. The protocol used in the semi-quantitative PCR was the same to that used in the qualitative PCR, except for the addition of 2.5 U of Taq DNA polymerase and 1 µg of the clinical sample DNA. For the semi-quantitative PCR, samples containing 80000, 8000, 800 and 80 plasmid particles were tested simultaneously with the clinical samples. The PCR products were

subjected to electrophoresis in 2% agarose gel and the amplicons were visualized by UV light and photographed with the Science 1D digital came (Kodak, USA). The amplicon band densities were determined using the DC120 Digital Access software (Kodak, USA).

The CMV load of the clinical samples was determined by plotting the amplicon band density value of each sample into a graphic including samples containing 80000, 8000, 800 and 80 plasmid particles, as shown in Fig. 1.

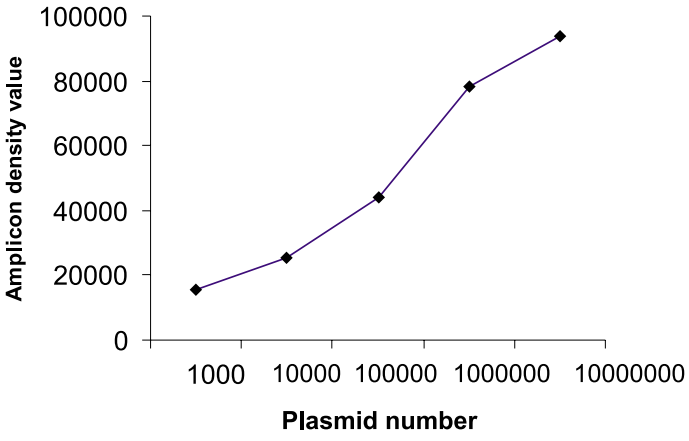

Fig. 1 - Graphic of density values of amplicon bands obtained from 800, 8 000, 80 000, 800 000, 8 000 000 gB CMV plasmids in the semi-quantitative PCR.

Statistical analysis: Personal and clinical data as well as laboratory test results were confronted to results of the CMV qualitative and of the semi-quantitative PCR tests by using the Chi-square and the Fisher's exact test ($p < 0.05$).

RESULTS

Qualitative PCR: Sixty three (26.5 %) of the 237 randomly selected HIV infected individuals had CMV genome detected in the PBL. Nineteen (8%) of the 237 randomly selected HIV infected individuals had CMV genome detected in the plasma. Only 4 patients had CMV genome detected in both leukocytes and plasma (Fig. 2). The positive results were confirmed for all the clinical samples by nested PCR.

Only one (10%) patient of the 10 individuals presenting clinical manifestations suggestive of CMV disease had CMV genome detected in the leukocytes by qualitative PCR. None of these patients had CMV genome detected in plasma.

Semiquantitative PCR: CMV DNA load was determined in the 63 randomly selected HIV infected individuals having CMV genome detected in PBL by qualitative PCR (Fig. 3). The semi-quantitative PCR showed that in 10 cases (15.8%) the CMV loads ranged between 928 to 332 880 copies/µg DNA. The remaining 53 individuals had CMV load under 800 viral copies/µg DNA, the sensitivity limit of the method.

One patient from those presenting clinical manifestations suggestive of CMV disease had CMV genome detected in the leukocytes by qualitative PCR and showed a CMV load of 8 000 000 viral copies/µg DNA.

Personal and clinical data of patients having CMV genome detected by qualitative PCR: The average age of the 82 patients having CMV genome detected by qualitative PCR was 34.2 years, the average CD_4 lymphocyte count 279.96 cells/mm^3; and the average HIV load 10,000 copies/ml of plasma. Seventy patients were using high active anti-retroviral therapy. No significant association of patient age, gender, HIV load, CD_4 and CD_8 leukocyte counts and infection stage with CMV genome detection was observed. Likewise, none of the patients having CMV genome detected by qualitative PCR presented clinical manifestations associated to CMV disease and no association of CMV

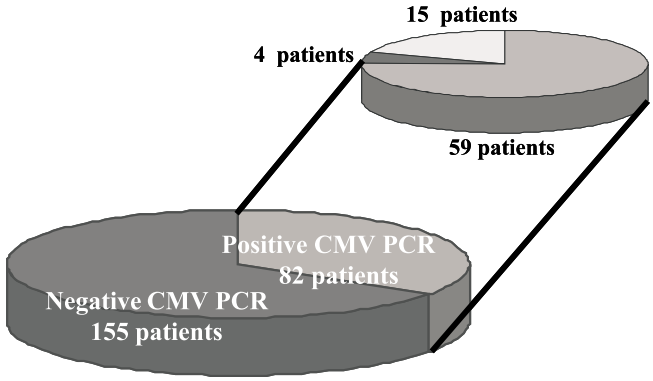

Fig. 2 - Positivity of the CMV qualitative PCR in serum, buffy coat and in both samples in the group of 237 HIV infected patients. The small pizza highlight 59 patients with positive test only in the buffy coat, 15 patients with positive test only in the serum and 4 patients with positive test in both, buffy coat and serum.

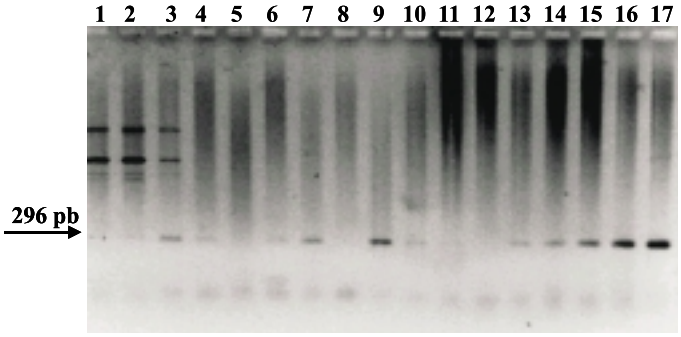

Fig. 3 - Electrophoresis in 2% agarose gel showing: **in columns 12 to 17,** amplicons with 296 bp of the plasmid titration (ten-fold dilutions; 17 = 8 X 10^6 plasmids; 16 = 8 X 10^5 plasmids; 15 = 8 X 10^4 plasmids; 14 = 8 X 10^3 plasmids; 13 = 8 X 10^2 plasmids, and 12 = 80 plasmids. The amplicon band is not observed in column 12 due to the low plasmid quantity that is below the sensitivity of the test. Patient samples were run in **columns 1 to 11,** showing amplicon bands in columns 3 (15 768 CMV copies/µg DNA); 7 (8 875 CMV copies/µg DNA), and 9 (332 880 CMV copies DNA/µg). In columns 1, 2, 4, 6 and 10, are shown amplicon bands with a lower density than the sensitivity of the test. In columns 5, 8 and 11, amplicon bands cannot be observed.

load with the use of anti-retroviral therapy and death evolution was observed.

Personal and clinical data of patients having a determined CMV load: Personal and clinical data of the patients having a determined CMV load are shown in Table 2. These patients had an average of CD_4 lymphocyte count of 384.9 cells/mm^3 and HIV load average of 76,000 copies/ml of plasma. No significant association of patient age, gender, HIV load, CD_4 and CD_8 leukocyte counts, and infection stage with CMV load was observed. Likewise, none of these patients presented clinical manifestations associated to CMV disease. No association of CMV load with the use of anti-retroviral therapy was observed. A significant association between deadliness rates and CMV load was observed ($p < 0.005$) in the patients. However, the death diagnosis of these cases, nosocomial pneumonia and intestinal acute perforation, could not be included as CMV disease clinical manifestations.

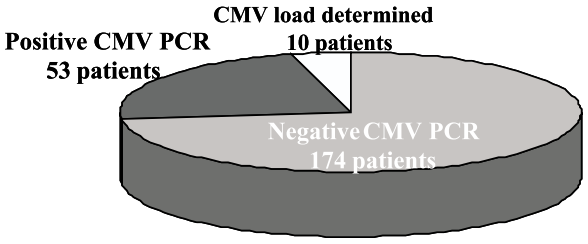

Fig. 4 - Positivity of the CMV qualitative PCR tests in the buffy coat of the 237 HIV infected patients, highlighting 10 cases having a determined CMV load and other 53 positive patients whose CMV load was below to the sensitivity test.

In the group of individuals presenting clinical manifestations, the patient presenting high CMV load (8 000 000 viral copies/µg DNA) also showed the lowest CD_4 lymphocyte count, 0.5 cells/mm^3, and came to death.

DISCUSSION

A 95% prevalence of IgG antibodies to CMV in pregnant women of Ribeirão Preto was reported by YAMAMOTO *et al.* in 1999[18]. This high prevalence of CMV infections in the adult population is probably also observed in the HIV infected population. However, despite a high incidence of CMV infections a great decrease of CMV disease it is probably due to the raised use of HAART[4,14,15,17]. In our study, CD_4 lymphocyte counts below 100 cells/mm^3 were observed in a small number of the HIV infected patients having CMV genome detected (12/82, 13%) and only one of these cases was confirmed as having CMV disease. Our data corroborate those reported by the other authors[4,14,15,17].

PCR method is considered a valuable tool for diagnosis in virology due to a high sensitivity that allows detection of very low amounts of DNA in clinical samples. CMV genome can be easily detected by PCR in PBL samples[14]. This method has been used in our laboratory for the diagnosis of CMV infections, testing urine, blood of children and kidney transplant recipients, with suitable performance[1,18].

We presented a transversal study using the blood samples of 237 HIV infected patients. CMV DNA was detected in 34% of these cases by qualitative PCR. Likewise, Marin in personal communication, using the same PCR method detected CMV DNA in 27.3% of 37 kidney transplant recipient patients in Ribeirão Preto. Our results show a higher frequency of CMV infections when compared to those observed by the other authors. PELLEGRIN *et al.* in 1999[14], found CMV genome in 19.8% of 58 HIV infected French patients.

Table 2
Clinical and laboratorial data of the 10 HIV infected patients that had a determined CMV load

Patient	Age	Gender	HIV-load Copies/ml	CD_4 cel/mm^3	CD_8 cel/mm^3	*Stage CDC-93	CMV load Viral copies/µg DNA	Opportunistic infections Treatment	Evolution
JCD	37	M	12 000	285	643	C-3	3311	Neuro-cryptococcosis HAART**	Death
MRLS	34	F	4 900	239	494	A-3	21837	None OI**** HAART	Stable
ERM	31	F	55 000	72	380	A-3	14278	None HAART	Stable
RB	34	M	260 000	281	996	C-3	31167	Retinitis HAART	Death
DL	45	M	1 800	269	1434	C-3	26866	Strongyloidiasis HAART	Stable
CCJ	33	M	6 700	887	1432	C-3	332880	None OI No treatment	Stable
VSF	33	M	200 000	628	1948	A-1	15,768	No treatment	Death
MATA	19	M	73 000	522	627	A-1	9,918	No treatment	Stable
AMTB	36	M	140 000	242	946	C-3	928	Histoplasmosis HAART	Stable
JPS	41	M	1 800	424	1391	A-3	8,875	HAART	Stable

*Clinical classification; **HAART-Anti-retroviral therapy; ***ND-not done; **** OI-Opportunist infection.

CMV genome was detected in the plasma of only 8% of our HIV infected patients. This positive result rate is not higher than that reported by PELLEGRIN *et al.*[14] in the plasma of French HIV infected patients, 13%. The presence of CMV DNA in plasma shows that the virus is replicating, despite the lower sensitivity of the test when using plasma for CMV genome detection if compared to blood leukocytes. The diagnosis of CMV replication in the blood is named active infection, it is known as a high-risk condition for the development of CMV disease. In the subgroup of 19 patients having CMV genome detected in plasma, the virus was observed in the plasma but not in the PBL for 15 cases. This intriguing fact could be a result of CMV replication in other sites (mainly endothelial cells) rather than PBL[11,14].

CMV DNA was detected in PBL of 26.5% (63 patients) of the HIV infected cases, showing higher sensitivity when compared to the CMV detection in plasma. Blood leukocytes are known as traditional sites for CMV replication[10], and besides the amount of DNA in PBL is higher than in plasma, even using ion change column to extract DNA from plasma. Based on these results, PBL should be chosen as the most suitable blood component for CMV genome detection.

The sensitivity of the semi-quantitative PCR tested in the present work was of 800 CMV copies/µg of DNA, which is probably adequate for the diagnosis of CMV active infection and CMV disease.

In the group of 237 HIV infected patients chosen randomly only 10 had CMV load determined with rates of 928 to 332 000 CMV copies/µg DNA. Despite being active infection cases, CMV disease was not observed during the follow-up of these 10 patients. We associated the absence of CMV disease with the use of HAART in 70% of the studied patients. Most of the patients having CMV genome detected, 53 individuals, showed a low CMV load, under 800 viral copies/µg DNA, probably due to a partial control of the CMV replication by the immune system, particularly in those patients using antiretroviral drugs.

In the group of 10 individuals presenting clinical manifestations, one patient had a severe form of CMV disease causing hepatitis, retinitis, and lung infiltrates. This patient with a high CMV load, 8 000 000 viral copies/µg DNA, also showed a very low CD_4 lymphocyte count, 0.5 cells/mm^3, and came to death, despite usual treatment to CMV[7]. The diagnosis of CMV disease of this patient was also corroborated by the presence of cytomegalic inclusions in hepatocytes. The absence of CMV active infection in 90% of our patients presenting clinical manifestations suggestive of CMV disease show clearly the low specificity of the clinical suspicion only based on clinical manifestations and that those signals and symptoms can also occur in other infections of AIDS patients.

In short, qualitative PCR followed by confirmatory nested-PCR with gB primers allowed the screening of CMV infections in HIV infected patients. The CMV genome was detected in an expressive amount of patients. However, qualitative PCR was not able to diagnose or to discriminate CMV active infection or disease. The semi-quantitative PCR was able to detect CMV load in 11 patients. Despite being active infection cases, only 1 of these patients presented CMV disease. This patient presented a very high CMV load and a severe form of disease. The difficulty on finding CMV disease cases in the 247 HIV infected cases is probably due to the use of HAART by these patients which increase the number of CD_4 cells and improves their cellular immune responses. Further studies are necessary in order to confirm the usefulness of this CMV semi-quantitative PCR in HIV infected patients.

RESUMO

Diagnóstico de infecção por CMV em pacientes infectados pelo HIV utilizando PCR qualitativa e quantitativa

Uma alta incidência de infecção pelo citomegalovirus (CMV) é observada no Brasil. Este vírus é responsável por significante morbi-mortalidade entre pacientes infectados pelo vírus da imunodeficiência humana (HIV). Neste estudo, mostramos a aplicação de uma PCR quantitativa para determinar a carga de CMV nos leucócitos do sangue periférico e no plasma de 247 pacientes infectados pelo HIV. As amostras clínicas foram previamente testadas por uma PCR qualitativa e confirmadas por uma *nested*-PCR para posteriormente serem quantificados. Contagem de linfócitos T CD_4, carga viral do HIV e sintomas de doença citomegálica foram correlacionados com carga de CMV. O genoma de CMV foi detectado nos leucócitos do sangue periférico em 82 de um total de 237 (34%) pacientes, em 10 destes a carga de CMV foi detectada variando de 928 a 332880 cópias virais/µg de DNA. Nenhum destes 237 pacientes desenvolveu manifestação clínica sugestiva de doença pelo citomegalovírus. Para outro grupo de 10 pacientes infectados pelo HIV e selecionados com base na suspeita de doença pelo CMV, o genoma do CMV foi detectado em apenas um caso. Este paciente apresentava alta carga de CMV (8 000 000 de cópias virais/µg de DNA), desenvolvendo forma disseminada de doença pelo CMV o que incluiu hepatite e retinite. Nossos resultados foram fortemente influenciados pelo impacto da terapia antiretroviral que reduziu a incidência da viremia por CMV, bem como a ocorrência de doença citomegálica em pacientes infectados pelo HIV.

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