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Cytomegalovirus Infections in Patients with HIV/AIDS in a Unit of Health of the Amazonian Region, Belém, Pará, Brazil

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Abstract

Introduction: With the increase in prevalence of HIV/AIDS in the world, infection by CMV became a serious public health problem because of the immunodeficiency caused by HIV and the reduction of TCD4+ cells.

Objective: Clinical-epidemiological and laboratory aspects of patients were evaluated, which were admitted in a public hospital in the Amazon Region, Belém-Pará, Brazil.

Methods: We collected clinical and epidemiological data by questionnaires and medical records, and whole blood for detection of anti-CMV antibodies by ELISA; and the method of Polymerase Chain Reaction in Real Time (qPCR) for detecting viral load.

Results: The socioeconomic data indicated high frequency of individuals with incomplete level of basic education (35.3%) and low income (57.7%). Important comorbidities were found by using medical records; pulmonary tuberculosis (19.9%), toxoplasmosis (19.5%), extrapulmonary tuberculosis (14.5%) and diarrheal syndrome (14.1%) occurred more frequently. According to the serological analysis it was observed that only 2.1 of patients had acute infection profile (IgG+IgM+), while in qPCR more than 50% of patients had high viral load (M =107,479.48 copies/ml). During this study, 49 patients died, 63.3% were co-infected by HIV/CMV detected using molecular method. It was observed the highest occurrence of CMV-infected individuals when the TCD4 lymphocytes were <100cells/mm^3. There were significant differences between molecular data and serological results (Z=12.98, p<0.0001).

Conclusions: Molecular methods are the most appropriate technique to help in the clinical diagnosis of secondary CMV infection in immunodeficiencies and the reduction of CD4+<100/mm^3 cells is an important risk factor that predisposes people with HIV/AIDS to opportunistic infections.

Keywords: Cytomegalovirus; Human immunodeficiency virus; Acquired immunodeficiency syndrome immunodeficiency; Co-infections; Load viral

Introduction

Following the emergence of Acquired Immunodeficiency Syndrome (AIDS), opportunistic infections caused by various biological agents became one of the main public health concerns relative to individuals living with the immunodeficiency virus HIV/AIDS. Antiretroviral therapy (ART) is able to restore the patients' immunity, increasing the number of CD4+ T lymphocytes to their normal values, and it is also able to reduce the HIV viral load [1-3]. In spite of the therapeutic and preventive measures available for the control of infection, epidemiological data indicate that a significant number of individuals are still infected by HIV, particularly youths [4,5]. In Brazil, infection has exhibited a remarkable decline in the Southern and Southeastern areas as a function of prevention and the treatment of HIV carriers. In contrast, the Northern and Northeastern areas are a cause for concern due to the increasing incidence of cases [4] and the high morbidity and mortality rates associated with opportunistic infections [6-9].

Cytomegalovirus (CMV) stands out among the main causes of secondary infections to AIDS because it seemingly acts as an inducer or co-factor in the progression of HIV infection pathogenesis [10-13]. Because it accelerates the progression of AIDS and the occurrence of death, CMV infection is considered to be one of the main causes of morbidity and mortality among immunosuppressed individuals [14-17].

Infection by CMV can be acquired early in life, between the end of infancy and beginning of adolescence, and its prevalence increases in adulthood. The virus remains in the organism indefinitely [18], particularly in salivary gland cells and leukocytes, which are considered to be infection sites [19]. Epidemiological factors such as high prevalence [20], and the presence of CMV in various biological fluids, such as cervix discharges, sperm, mother's milk, oropharyngeal discharges, tears, and blood [10,21-23] facilitate its transmission by means of unprotected sexual intercourse, sharing of contaminated injection materials, blood transfusions, and organ transplantation [14]. It is notable that both HIV and CMV exhibit the same paths of transmission, in addition to having clinical characteristics in common [24] 002E Factors such as a drastic decrease of the CD4+ T lymphocyte count (<100 cells/mm^3), with a consequent loss of immune resistance, as well as the long persistence of CMV in the organism [25,26], contribute to the high morbidity and mortality rates exhibited by patients with HIV/AIDS. Under such circumstances, the patient becomes more vulnerable to the opportunistic diseases caused by CMV,

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especially by reactivation of its latent form [27-29] When ART is not performed, infection by CMV can be associated with severe clinical manifestations [30] affecting significant organs and systems, such as the retina (retinitis), lung (interstitial pneumonia), liver (hepatitis), heart (carditis), gastrointestinal system (gastroenteritis, colitis, and esophagitis with ulcerations), and nervous system (encephalitis) [31-33], which increase the risk of death [7,25,34].

According to Matos et al. [35], there is an inverse correlation between clinical manifestations and the CMV viral load. Fielding et al. [7] found a strong correlation between high numbers of CMV copies and the mortality of HIV+ carriers, not restricted to individuals with advanced stages of AIDS. To better understand the actual dimension of CMV infection within the context of the opportunistic infections exhibited by individuals with HIV/AIDS, an epidemiological, clinical, and laboratory study of CMV was performed with inpatients living with AIDS at a reference hospital for infectious and parasitic diseases in the city of Belém, state of Pará, Northern Brazil.

Materials and Methods

The present investigation was a prospective cross-sectional study conducted from December 2010 to December 2011. A total of 241 in patients with HIV/AIDS at João de Barros Barreto University Hospital (HUJBB, Federal University of Pará), in the city of Belém (Pará State, Brazil), were tested for infection by CMV. The study was approved by the Human Research Ethics Committee of Evandro Chagas Institute under a Certificate of Presentation of Ethical Appraisal - CAAE-5618.0.000.072-09. The study included patients aged 18 years old or older from both genders. The participants responded to clinical-epidemiological questionnaire seeking information on their personal and socioeconomic data, past pathological history, and clinical and laboratory data. Further information was extracted from the participants' clinical records. Then, a 10 ml blood sample was collected and divided into two aliquots: a 7 ml aliquot was placed in test tubes without anticoagulants to separate the serum, and a 3 ml aliquot was placed in test tubes containing EDTA for use in molecular analysis.

The blood samples were processed at the Laboratory of Virology and Molecular Biology of the Environment Unit, Evandro Chagas Institute, Secretariat of Health Surveillance and Health Ministry. Anti-CMV IgM and IgG antibodies were investigated by means of ELISA, Diassorin (Saluggia, Italia) and viral DNA to detect and measure the viral load by means of qPCR.

The viral genomic material (DNA) was extracted by the silica method, using a kit manufactured by Laboratory G&E®. Then, the DNA was quantified using a spectrophotometer Nano Drop 2000 (Wilmington, DE, USA, followed by CMV viral load detection using a kit manufactured by Laboratory GE Healthcare (Buckinghamshire, UK), which includes three reaction plates, optical seals, four vials of AmpliPROBE™, four vials of AmpliMIX, four vials of AmpliMASTER, four plasmid solutions with known concentrations (10^5, 10^4, 10^3, and 10^2 copies/5 µL) for the purpose of quantification, and four vials with plasmid solutions of the human beta-globin gene to serve as an internal control in the quality analysis of viral DNA extraction and amplification.

The plaque reaction was controlled using the standard 10^5 point, cT of 25, and R2 varying from 0.99 to 1.00. The qPCR was performed following the kit manufacturer's instructions, and the target-segment of gene MIE was amplified. Real-time PCR was performed using the 7500 *Real Time PCR System*, by *Applied Biosystems*®, and the laboratory sent the results to the hospital for inclusion in the participants' clinical records to allow appropriate therapeutic measures against CMV infection to be started.

Results

The epidemiological data in the questionnaire showed that 143 (59.3%) participants resided in Belém and 96 (39.9%) in other towns of the state of Pará, while only two (0.8%) came from other states. The sociodemographic data showed that 79.3% (N=191) of the sample were male and that the ratio of infection cases per gender was 3.8 males to 1 female. The Figure 1 presents the absolute frequency of the volunteers' gender and marital status. Approximately 68.9% of the sample reported being single (N=166), and the age ranges most frequently found were 26-30 years old (20.3%) and 31-35 years old (17.8%). The educational level most frequently reported was unfinished elementary school (N=35.3%), and the monthly family income range most frequent reported corresponded to the equivalent of once to twice the minimum wage (N=139; 57.7%).

Figure 1, Frequencies of the volunteers' gender and marital status. Bethlehem, Pa, Brazil (2010-11).

Among the risk factors reported by the volunteers, the ones that most significantly contribute to HIV/CMV comorbidity were assessed. Relative to the number of sexual partners, 66 (27.4%) participants reported that they had not had sexual intercourse in the past six months, and 50 (20.8%) reported intercourse with three or more partners. Fifty-four (22.4%) volunteers reported that they did not use condoms before HIV infection, and 52 respondents (96.3%) started using condoms only after testing positive for HIV. Whole blood and/or component transfusions were mentioned by 83 volunteers (34.4%). Eighty-six participants (35.7%) reported the use of illegal drugs at some time in their life, of whom 87.2% (N=75) reported being users of non-injectable drugs and 12.8% (N=11) of injectable drugs. Only 14 volunteers (5.8%) reported having received body piercings, and 89 (36.9%) exhibited tattoos.

The data on the opportunistic diseases diagnosed during the period under study are described in Table 1; these conditions include pulmonary tuberculosis (TB) as the most frequent, followed by

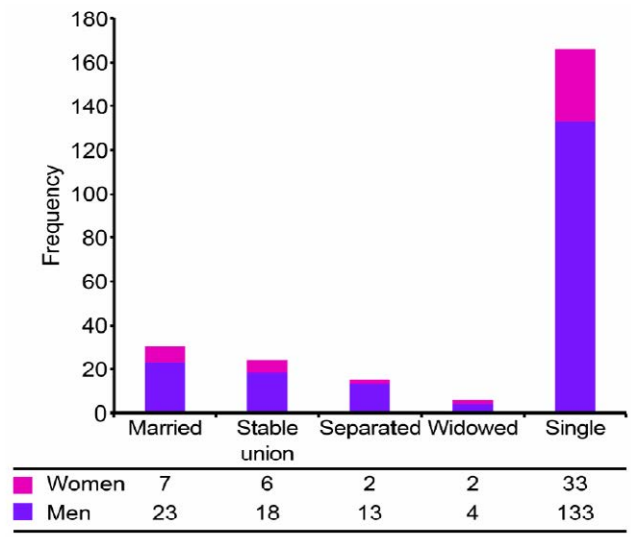

Figure 1: Frequencies of the volunteers' gender and marital status. Bethlehem, Pa, Brazil.

Opportunistic diseases	N	Freq.
Pulmonary tuberculosis	48	19.9%
Cerebral toxoplasmosis	47	19.5%
Extrapulmonary/disseminated tuberculosis	35	14.5%
Chronic nonspecific diarrhea	34	14.1%
Pneumocystis jiroveci pneumonia	10	4.1%
Extrapulmonary cryptococcosis	7	2.9%
Cytomegalovirus retinitis	7	2.9%
Esophageal candidiasis	6	2.5%
Bacterial pneumonia	6	2.5%
Kaposi's sarcoma	5	2.1%
Disseminated histoplasmosis	5	2.1%
Recurrent oral/vaginal candidiasis	4	1.7%
Chronic intestinal cryptosporidiosis	2	0.8%
Other	54	22.3%

Table 1: Opportunistic diseases registered in the clinical records of patients with HIV/AIDS. Belém - Pa, Brazil (2010-11).

ART	Cause of death				
	Hypovolemic shock	Septic shock	Gastrointestinal complications	Neurological complications	Respiratory complications
No	0	2	2	3	5
<6 m	0	6	2	5	7
6 to 12 m	0	1	0	0	0
1 to 5 y	1	0	1	1	2
5 to 10 y	0	0	0	1	6
>10 y	0	0	1	1	2
Total	1	9	6	11	22

Table 2: Cause of death of individuals living with HIV/AIDS according to length of ART, Belém - Pa, Brazil (2010-11).

Laboratory data	N	CMV viral load			Negative
		<1,332	1,332 – 66,660	>66,660	
CD4+ T cells					
<100/mm^3	46	13	13	06	14
100 to 200/mm^3	19	08	04	00	07
>200/mm^3	28	03	07	01	17
Not reported	148	35	36	13	64
HIV viral load					
<500,000 copies	42	10	10	02	20
50,000 to 500,000 copies	38	11	12	01	14
>500,000 copies	13	03	02	04	04
Not reported	148	35	36	13	64

Table 3: Correlation of CMV viral load with HIV viral load and CD4+ T cell count in patients living with HIV/AIDS. Belém, Pa, Brazil (2010-11).

cerebral toxoplasmosis, extrapulmonary TB, and nonspecific diarrhea. CMV infection was less frequent (2.9%) according to the information registered in the participants' clinical records.

Sixty participants (24.9%) were not under ART, 85 (35.3%) had started ART about six months earlier, 65 (26.9%) had been under treatment for more than one year, and 31 (12.9%) had been for more than 10 years. The correlation between clinical manifestations and duration of ART showed that the frequency of clinical manifestations was lower among the patients using ART for six to 12 months. In addition, a negative correlation was found between the appearance of clinical manifestations and length of treatment, whereby the longer treatment corresponded to a lower tendency for clinical manifestations to appear (linear correlation, r=-3.6; p=0.0207). The anti-CMV serum tests showed that 240 (99.6%) participants had been in contact with CMV at some time in their life (IgG+/IgM-), and 5 (2.1%) tested positive for IgM antibodies, indicating recent CMV infection. Real-time PCR detected viral DNA in 134 participants (55.8%), with a viral load varying from 14 to 4,764,538 copies/ml (average of 107,479.5 copies/ml). There were significant differences between the molecular data and serological results (Z=12.98, p<0.0001). Forty-nine participants died within the study period as a result of different complications, with respiratory problems being the most frequent cause, especially among patients who had used ART for less than six months (Table 2). Among the volunteers who died, one tested positive for IgM antibodies and had CMV viral load of 8,849.14 copies/ml. That individual's clinical records did not report the HIV viral load or CD4+ T cell count, and the cause of death was complications of chronic nonspecific diarrhea and gastroesophageal ulcers. The other 48 participants who died (97.9%) exhibited serological profiles of past infection (IgG+/IgM-). CMV DNA was detected by means of qPCR in 33 (68.8%) of them. Among the participants who died, 32 (65.3%) had used ART for less than six months (Table 2). Joint analysis of the clinical, serologic, and molecular data of the participants with recent infection (N=5) indicated that 1 (1.7%) had clinical manifestations compatible with diarrheic syndrome (diarrhea, fever, headache, and dyspnea), HIV viral load (VL) between 50,000 and 500,000 copies/ml, CMV VL of 34,384.5 copies/ml, and CD4+ T cell count <100 cells/mm^3. Two other participants exhibited a febrile syndrome (fever and asthenia), one of whom had a CMV VL of 8,997.0 copies/ml. The laboratory results (serology and qPCR) of the other participant were inconclusive, whereas the HIV VL and CD4+ T cell count were not reported in either case. The fourth volunteer tested positive for IgM and exhibited general clinical and neurological manifestations (fever, sweating, asthenia, lymph node affection, headache, paresthesia, mental confusion, and seizures) and a CMV VL of 10,478.2 copies/ml. The last patient in this group exhibited a CMV VL of 8,849.14 copies/ml and clinical manifestations of diarrheic syndrome and oropharyngeal ulcers. Also in this case, where the cause of death was gastrointestinal problems, the HIV VL and CD4+ T cell count were not reported.

In fact, data on the HIV VL and CD4+ T cell count were available in only 93 cases. Joint analysis of those data with the corresponding CMV VL values (Table 3) indicated an inverse correlation trend between CMV VL and CD4+ T cell count (X^2=5.7; A=-9.9; p=0.0172); that is, lower CD4+ T cell count corresponded to higher predisposition to CMV replication. The correlation between an HIV VL greater than 50,000 copies/mm^3 and positive detection of CMV by means of qPCR did not exhibit statistical significance (χ^2=0.912; A=3.21; p=0.339).

Discussion

The present study found a high prevalence of antibodies indicating past infection among the participants (N =240; 99.6%). This finding, together with the epidemiological results, is compatible with the epidemiological profile of CMV in developing countries, where the seroprevalence rates are greater than 80% [3,24]. The age range that exhibited the highest prevalence of IgG antibodies was between 26 and 30 years old (20.3%), while the frequency of those antibodies decreased together with age. These findings point to an inverse correlation between the frequency of IgG antibodies and age, which contradicts the results reported by Junqueira et al. [18], according to which the prevalence of antibodies not only increases but bears a direct correlation with age and sexual activity.

Significant risk factors associated with HIV and CMV transmission were identified. Approximately 37% of the sample reported whole

blood and/or component transfusions, use of illegal injectable drugs, body piercings, and tattoos. Factors related to sexual activity play an important role in the transmission of HIV and CMV. In our study, 54 (22.4%) participants reported not having used condoms before HIV infection, and 20.8% reported having had intercourse with three or more partners, although they did not state whether that occurred after testing positive for HIV, nor whether they had used condoms.

In regard to other risk factors related to the morbidity and mortality of co-infected patients, the correlation between HIV VL (>50,000 copies/mm^3) and positive detection of CMV by means of qPCR was not statistically significant ($\chi^{2=}$0.912; A=3.21; p=0.339). That is, high HIV VL did not behave as a predictive risk factor for infection by CMV in patients with HIV/AIDS. In contrast, HIV/CMV coinfection induced a two-fold increase of the relative risk of death, resulting in a ratio of 1:11 individuals (RR=1.66; p=0.0449). Those findings corresponded to the participants who died, as that was the group with the highest frequency of positive results on qPCR, in addition to an average CMV load of 107,479.5 copies/ml. According to Fielding et al. [7], high CMV VL is a significant risk factor for death in patients living with AIDS, whereas the association between CMV and mortality is not restricted to individuals in advanced stages of AIDS. Our data, together with those reported by Gandhi and Khanna [14], Tsutsui et al. [17], and Khan et al. [15] indicate that CMV is one of the main causes of morbidity and mortality among immunosuppressed individuals. The CD4+ T cell count is a further risk factor worthy of consideration, as the greater number of individuals in whom qPCR detected CMV exhibited < 100 cells/mm^3. This finding shows that CD4+ T cell counts below that reference value represent an important risk factor for CMV infection in patients with HIV/AIDS.

Based on the data registered in the participants' clinical records, the most frequent opportunistic infections were pulmonary TB, neurotoxoplasmosis, extrapulmonary TB, and chronic nonspecific diarrhea. CMV (retinitis) corresponded to 2.9% of the opportunistic diseases. This finding contradicts the results of qPCR, which detected CMV DNA in the peripheral blood of 55.8% of the sample. In contrast, the data on CMV collected from the clinical records agree with the serological results (2.1%), thus suggesting that diagnosis was most likely established by means of serological tests. On these grounds, we might infer that CMV infection was underestimated in the investigated sample of immunosuppressed individuals with HIV and that a more appropriate method should be used for the diagnosis of CMV infection in immunodeficient patients. According to Mello et al. [32], qPCR is the best diagnostic method available to monitor opportunistic infections by CMV.

Relative to the serological results, it is worth noting that almost 100% of the sample exhibited anti-CMV IgG antibodies. Those antibodies behave as a humoral immune factor of protection against countless infections in immunocompetent individuals, which does not occur among immunodeficient patients, especially when the CD4+ T cell count is less than 100/mm^3, as was the case of the investigated sample. These findings corroborate the significant role that the immune cell response plays in opportunistic infections. Several authors found recurrent CMV infection despite the presence of anti-CMV IgG antibodies in patients with chronic or transient immunosuppression [14,26,27,35]. According to Yamamoto et al. [29], and Terra et al. [28], CMV infections in immunosuppressed patients occur due to viral reactivation, which may have occurred in the present sample, as shown by the high prevalence of participants with the IgG+/IgM- serological profile.

Among the clinical manifestations exhibited by individuals with HIV/AIDS co-infected by CMV, retinitis is the main disorder related to the latter [3]. However, retinitis was not the main clinical disorder affecting the participants in our study. This finding may be attributed to underdiagnosis of retinitis, as 80.9% of the volunteers reported not having performed ophthalmoscopy, and 90.8% among them exhibited positive results on qPCR.

Relative to the most frequent clinical manifestations, general (66.0%) and gastrointestinal (58.1%) symptoms predominated. According to Marques Jr. et al. [36], CMV is the most common etiological agent of colitis in HIV-infected patients, being the cause of up to 45% of the cases of severe diarrhea and other gastrointestinal disorders, such as esophagitis, dysphagia (20.9%), and odynophagia (18.4%). Also according to Lima et al. [16] enterocolitis by CMV is one of the most frequent clinical disorders exhibited by patients with HIV, second only to retinitis. In our study, some participants exhibited nonspecific diarrhea, eventually also with esophageal ulcers. The CMV viral load was greater than 1,332 copies/ml, which is the minimal standard value of the qPCR technique, in 44.9% of those volunteers, thus suggesting that CMV was the cause of some of the gastrointestinal disorders found.

The presence of clinical manifestations in patients using ART might point to the occurrence of the immune reconstitution inflammatory syndrome (IRIS). Nevertheless, that hypothesis could not be investigated due to the lack of sufficient data on the participants' CD4+ T cell count before and after the onset of ART. In the present sample, clinical manifestations were found in a small number of the patients who used ART for six months or more than five years. This finding might be attributed to therapeutic failure associated with factors such as HIV resistance to antiretroviral drugs; episodes associated with difficulties in adhering to ART, which are complex and multifactorial; and immunosenescence due to long-standing HIV infection and chronic CMV infection [37-39]. The persistent presence of HIV and CMV antigens triggers a phenomenon known as "T cell exhaustion," which is characterized by the functional loss of T cells and cytokine release, resulting in inefficacy of the immune response. In contrast, the clinical manifestations were considerably reduced in the patients using ART. Statistical analysis indicated a favorable correlation between longer ART and reduced clinical manifestations (r=-3.6; p=0.0207), which was also found by Brantsaeter et al. [1], who reported that institution of ART bears direct correlation with reduction of the incidence of CMV infection.

Our findings show that the clinical-epidemiological features of CMV infection are maintained over time in groups characterized by low socioeconomic levels and in immunosuppressed individuals. CMV still plays a relevant role in the morbidity and mortality of immunodeficient individuals, mainly when associated with HIV, and low CD4+ T cell counts. CMV infection must be included in the group of opportunistic and parasitic diseases monitored in individuals with HIV/AIDS, especially because it is currently underestimated, while it plays a relevant role in the lethal outcome of the disease. It is worth further stressing the relevance of molecular biological tests in the diagnosis of subclinical infection and viral reactivation, as this type of infection is most frequent in immunosuppressed individuals.

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Author's contributions

DFLS: Conceived, coordinated and designed the experiments; Wrote the paper

LMFA: Performed the experiments

NFS, MMM: collected the epidemiological data and blood samples

FESS: performed the statistical analyses

JLSAJr, TVRS: elaborated the questionnaire and database

R.S.M: performed the final revision of the paper and of the analyses

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Refereneces

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