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The Prevalence of Human Cytomegalovirus Viremia among HIV-1 Infected Individuals Undergoing Antiretroviral Therapy

Farag A. Bleiblo[1,2*], Souad A. M. Moftah[3], Madiha W. M. El-Awamie[1], Samira F. M. Bo Hagar[4], Asma A. Elamari[4], Aida M. M. Elakeili[5], Aseem Kumar[2]

[1]Department of Microbiology, Faculty of Science, University of Benghazi, Benghazi, Libya
[2]Department of Chemistry and Biochemistry and the Biomolecular Sciences Programme, Laurentian University, Sudbury, Canada
[3]Department of Physiology, Faculty of Medicine-Al-Marj Campus, University of Benghazi, Benghazi, Libya
[4]Department of Botany, Faculty of Science, University of Benghazi, Benghazi, Libya
[5]Department of Biology, College of Education, University of Benghazi, Benghazi, Libya
Email: *fx bleiblo@laurentian.ca

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Abstract

HIV infection is an emerging health issue in Libya, particularly among young adults. Human cytomegalovirus (HCMV) is a prevalent infectious agent that presents with subclinical and fatal diseases in immunosuppressed individuals including HIV-infected individuals. Although the impact of HCMV infection in HIV-positive patients is well documented in several regions, epidemiologic estimates concerning HCMV co-infection among HIV-infected individuals remain limited in Libya. Hence, this cross-sectional study was undertaken to derive data regarding the prevalence of active HCMV viremia among HIV-infected individuals undergoing antiretroviral therapy (ART) from Libya. A total of 90 consented HIV-infected subjects followed by the National Center for Disease Control (NCDC) of Benghazi/Libya were recruited in this study and investigated for HCMV-IgG, HCMV-IgM specific antibodies, detection of HCMV lower matrix phosphoprotein (pp65) antigen, and detection of HCMV-DNA using qPCR to assess the prevalence of HCMV viremia. We determined that 77 (85.56%) of subjects were seropositive for HCMV-IgG antibodies, whereas the seropositivity for HCMV-IgM was 3.33% (3/90 subjects). Our results also revealed that 4.44% (4/90) of participants had viral antigenemia based on the laboratory diagnosis of HCMV-pp65. Regarding the PCR, we were able to detect the DNA of HCMV only in 3/90 subjects (3.33%) suggesting an active viremic condition. The detection of HCMV DNA along with the HCMV-pp65 in HIV-positive individuals highlights the necessity of early diagnosis to manage the progression of the disease. Furthermore, we highly recommend the use of anti-HCMV therapy in viremic individuals in

combination with ART to reduce the burden of HCMV complications.

Keywords

HCMV, HIV, IgG, IgM, qPCR, Viremia, Prevalence

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus distributed worldwide. This virus is the most common infectious cause of birth defects and congenital diseases, the most significant and difficult opportunistic pathogen affecting immunocompromised patients [1] [2]. HCMV infects an overwhelming majority of the population, transmitted efficiently throughout life and universally through contact with bodily secretions. After the initial acquisition of HCMV, the virus replicates and causes a systemic infection, sometimes detected as a leukocyte-associated viremia, and disseminates to secretory organs such as salivary glands and kidney where replication produces virus found in secretions [3]. Viruses may be shed in any body fluids, including urine, saliva, tears, semen, and cervical secretions, and persistent shedding may continue for months to years, depending on age and immune status of the host [3] [4].

Despite potential antiviral drugs aimed to control the overall disease burden, the HCMV remains an important etiologic agent of opportunistic infections and disease in immunocompromised individuals following organ transplantation and immunosuppressive therapies, and genetic or acquired immunodeficiency [1] [2] [5]. Prior the use of ART, approximately 20% to 40% of adults with AIDS experienced HCMV disease. Among persons with HIV infection, the risk of HCMV disease is linked closely with the loss cellular defenses associated with a low CD4 T-cell count. Conversely, the use of ART has resulted in reconstitution or preservation of CD4 T-cell immune function in patients with HIV infection and has dramatically reduced the occurrence of HCMV viremia and disease. In the pre-ART era, the most prominent AIDS-associated HCMV disease was retinitis followed by esophagitis and colitis and less common clinical presentations such as encephalitis, peripheral neuropathy, pneumonitis, gastritis, and hepatitis [6]. HCMV remains a significant opportunistic infection in patients with HIV infection with significant immune impairment, and guidelines for preventing HCMV disease are focused on patients with fewer than 50 CD4 cells per ml of blood [7].

A recent comprehensive study in Libya has shown that HIV cases steadily increased within the Libyan population, particularly among those aged < 27 years. The prevalence of HIV infection varies geographically and has a variety of spatiotemporal patterns, with emergence of areas of high frequencies of HIV infection in specific regions and districts including the city of Benghazi [8]. Although much clinical and experimental progress has been made in identifying the implication of HCMV in HIV patients, the epidemiologic data concerning HCMV

co-infection in HIV-infected individuals has received little attention in Libya. Therefore, we aimed to determine the prevalence of HCMV viremia among treatment-HIV-infected subjects in Benghazi/Libya.

2. Materials and Methods

2.1. Patient Population and Study Design

This cross-sectional study was implemented among 90 HIV-infected individuals followed by the National Center of Infectious Diseases in Benghazi/Libya. We could not recruit the entire target population due to some technical issues, social concerns, and unavailability of some individuals. Therefore, we used subjective sampling technique with 95% confidence level to achieve accurate representative data. Informed consent was obtained from all patients and the protocol was approved by the Hospital's Ethics Committee. Demographic information including age, sex, marital status, literacy status, residential status, data about the onset of AIDS history were obtained by means of referring to medical records and personal interviews. All patients were confirmed HIV-infected adult individuals (aged > 18 years old) who attended the test centers.

Throughout their tenure on the units of NCDC, a 10 ml of blood sample was drawn from patients by vein puncture and placed into EDTA-treated tubes by trained medical personnel; it was left to stand at room temperature (20˚C - 25˚C) to allow for clotting, then the sera was separated by centrifugation 12,000 rpm for 5 minutes following WHO recommendations. Sera samples were stored at - 20˚C and later tested for cytomegalovirus antibodies by serological investigation. HIV-1 Viral load was first measured using the Abbott Real-time HIV-1 M2000rt (Abbott Laboratories, Abbott Park, USA).

2.2. Detection of HCMV IgG

Microplate-based enzyme-linked immunoassay (ELISA) was used for the qualitative detection of anti-HCMV IgG antibodies to cytomegalovirus in patients' sera according to the manufacturer's instructions (Autobio Diagnostics Co, LTD). Briefly, in the first step, sample and recombinant HCMV coated microwells are combined. During the incubation, the anti-HCMV antibodies present in the sample bind to the antigen coated in the wells. After the washing, in the second step enzyme-conjugate is added to the reaction mixture. The mouse anti-human IgG in the enzyme-conjugate is allowed to react with the anti-HCMV IgG attached to the solid phase in the first step. Then, a complex is generated between the solid phase, the anti-HCMV IgG within the sample, and the mouse anti-human IgG in the enzyme conjugate. Then, the substrate A and substrate B are added and catalyzed by this complex. The resulting chromogenic reaction is measured in absorbance. The color intensity, which corresponds to the amount of HCMV IgG antibodies present in the specimens, is measured with a microplate reader at 450/630 nm and the results were interpreted according to the manufacturer's instructions. Samples with optical densities 10% or more below

the cutoff were recorded as negative, those with optical densities between 10% below and 10% above the cutoff were equivocal, and all others were positive.

2.3. Detection of HCMV IgM

To determine if the patients have acute or primary infection, serum samples were assayed for HCMV-specific IgM using a HCMV IgM ELISA (Autobio Diagnostics Co, LTD) and the results were interpreted according to the manufacturer's instructions. Samples giving an absorbance less than the cut-off value were considered negative for the presence of HCMV-specific IgM antibodies whereas those giving an absorbance equal to or greater than the cut-off value were considered reactively positive.

2.4. Detection of HCMV Antigenemia

To quantitate the viral antigenemia, we performed Sandwich-ELISA to assay HCMV lower matrix protein pp65 in patient's sera according to the manufacturer instructions (Sung long Biotech). The microtiter strip plates provided with this assay are precoated with antibodies specific to HCMV pp65. Standards and samples were added to strip plate wells and combined to the specific antibody and then a horseradish HCMV pp65 (HRP)-conjugate specific for HCMV pp65 was added to each microelisa strip plate well and incubated. The TMB substrate solution was added to each after washing away the free components. The wells containing the HCMV pp65 and HRP-conjugated HCMV pp65 antibody appeared blue and then turned yellow after adding the stop solution. The optical density (OD) was measured by microtiter plate reader at 450 nm. The concentration of HCMV pp65 in the sample was calculated by comparing the OD of the sample to the standard curve according to the manufacturer instructions.

2.5. Extraction of DNA from Whole Human Blood

For the molecular detection of HCMV viremia, the whole human blood was collected in EDTA tubes and DNA extraction was carried out using DNA-Sorb-B according to manufacturer's instructions (Sacace Biotechnologies, Italy). The extracted DNA subsequently exposed to real time PCR (qPCR) test for quantitative detection of HCMV amplicons. Briefly, 300 µl of lysis solution was added to 100 µl of whole blood sample, vortexed, and incubated for 5 min at 65˚C and then centrifuged for 10 s. 300 µl of washing solution 1 was added to each tube, vortexed vigorously and centrifuged for 30 sec at 8000 g. Next, we added 500 µl of Washing Solution 2 to each tube, vortexed and centrifuged for 30 s at 8000 g and discarded supernatant from each tube. The pellet was resuspended in 50 µl of DNA eluent and then incubated for 5 min at 65˚C and centrifuged the tubes for 1 min at 12,000 g. The extracted DNA samples were stored at −20˚C.

2.6. Real Time PCR (qPCR) Test for Detection of HCMV

qPCR was performed on DNA extracted from whole human blood using HCMV

Real-TM Quant in accordance with the manufacturer's instructions (Sacace Biotechnologies, Italy). The test uses hot start for qualitative detection and quantification of human HCMV DNA in the clinical materials by using real-time hybridization-fluorescence detection. The amplification reaction was prepared as following: we prepared the mixture of PCR-mix-2-FRT and polymerase (TaqF) by transferring 30 µl of TaqF into 300 µl of PCR-mix-2-FRT and mixed by vortexing and then we mixed 5 µl of PCR-mix-2-FRT and Polymerase (TaqF) with 10 µl of PCR-mix-1-FRTHCMV. The total reaction volume is 25 µl (15 µl of the previously prepared qPCR mixture were added to 10 µl of DNA obtained from whole human blood or control samples. For the quantitative analysis, we added 10 µl of RNA-buffer to the tube labeled negative control of amplification. The amplification temperature profiles were created according to the manufacturer's recommendations. Briefly, the initial hot start was held at 95˚C for 15 mins and then the first cycling for 5 cycles was done at 95˚C for 5 s, 60˚C for 20 s and 72˚C for 15 s and then 40 cycles of 95˚C for 5 s, 60˚C for 30 s and 72˚C for 30 s. The results were interpreted by the software of the PCR instrument used by the crossing (or not crossing) of the fluorescence curve with the threshold line according to the manufacturer's instructions.

3. Results and Discussion

Several lines of evidence suggested that HCMV is a cofactor for rapid HIV-1 disease progression and associated with inflammation and immune activation in HIV infected patients [9] [10]. Therefore, the early diagnosis and intervention of opportunistic HCMV among HIV infected patients are very crucial for delaying HIV disease progression. Albeit the high HIV prevalence, there were only limited research performed concerning HCMV infection in African regions. Similarly, there is no documented report regarding HCMV co-infection among treatment HIV infected individuals from Libya. Methods to detect and quantify HCMV in clinical samples have been standardized into clinical routine as a valuable diagnostic tool in patients at high risk, including HIV infected patients. Hence, this study aimed to determine the prevalence of HCMV in an institution caring for HIV-infected individuals using serological and molecular approaches.

During this study, 90 consented HIV-positive patients were recruited during the study. The median age of the patient population was 22 (±24.9) years and about 46.7% (n = 42) of those were male participants and 48 (53.3) were females with approximately 1:1 ratio. Our results revealed that 77 (85.56%) out of these subjects were seropositive for HCMV-IgG as indicated in **Table 1**. Although not statistically significant, the seroprevalence of the HCMV IgG antibody was slightly higher in the female participants (54.55%) than in males (45.45%). According to the age-related prevalence of HCMV infection, the highest rate of HCMV-IgG antibodies was detected within the age group 20 - 30 years (84.41%) followed by those located within the age group 31 - 40 years (10.39%) as indicated in **Table 1**. In addition, we detected that 71 (92.21%) of the HCMV IgG

seropositive participants had HIV-1 viral load > 1000,000 copies/ml but the association was not statistically significant. Regarding the IgM antibodies, we found that only 3 subjects (3.33) were seropositive for HCMV-IgM indicating a recent viral infection. The HCMV-IgM seropositivity was more prevalent in age group 20 - 30 years and those subjects who had HIV-1 viral load greater than 10,000 copies/ml (66.7%) as indicated in Table 1. The associations between seropositivity of HCMV-IgG and HCMV-IgM were not statistically significant. Based on the laboratory diagnosis of HCMV-pp65, we determined that 4 (4.44%) patients had HCMV antigenemia. Of 90 participants, only 3 (3.33%) of them were shown to be positive for all three performed serological assays indicated the presence of recurrent infection.

Considering the clinical significance of HCMV DNAemia as an indicative for active HCMV infection and a marker of rapid HIV progression, we tested the patient blood samples for the presence of HCMV DNA. HCMV DNA was detected in only 3 (3/90, 3.33%) of the study participants. In terms of the sex distribution of the PCR positive samples, 66.7% (2/3) of them were females (Table 1). The viral load of HIV in these positive samples was also higher than 10,000 copies/ml. In addition, three of those PCR positive samples had positive HCMV-IgM and HCMV-pp65 results and there were statistically significant associations between these assays.

Several epidemiological studies on seroprevalence of HCMV infection in HIV-positive individuals has been published. In line with other reports from Ethiopia, Nigeria, and Sudan, the HCMV IgM seroprevalence in those studies was 12.4%, 11.7%, and 7.1% respectively [11] [12] [13]. However, the magnitude of the HCMV in our study is lower than those reported from these African countries. The Seroepidemiology variation in the prevalence of HCMV infection may rely on convenience samples from patients at a particular health institution,

Table 1. Comparison of IgG, IgM, pp65, and PCR results for detection of HCMV among the ART-HIV infected individuals.

Characteristics		Frequency (N) and Percentage (%)	Patients with Positive IgG		Patients with Positive IgM		Patients with Positive PP65		Patients with Positive HCMV DNA	
			(N)	(%)	(N)	(%)	(N)	(%)	(N)	(%)
Gender	Male	42 (46.7)	35	45.45	1	33.3	1	25	1	33.3
	Female	48 (53.3)	42	54.55	2	66.7	3	75	2	66.7
Age Group	20 - 30	76 (84.4)	65	84.41	2	66.7	2	50	2	66.7
	31 - 40	9 (10)	8	10.39	1	33.3	1	25	1	33.3
	41 - 50	5 (5.6)	4	5.20	0	0.00	1	25	0	0.0
HIV-1 Load (Copies/ml)	<1000	84 (93.3)	71	92.21	1	33.3	1	50	0	0.0
	1000 - 10,000	1(1.11)	1	1.30	0	0.00	1	25	1	33.3
	>10,000	5 (5.6)	5	6.49	2	66.7	2	25	2	66.7

geographical distribution and sensitivity of different assays used for detection of HCMV antibodies. Furthermore, the HCMV IgM seropositivity in the current study was also confirmed by HCMV-pp65 viremia assay and PCR results. The ability to detect the viral antigenemia and HCMV DNA in patients who were IgM positive might be due to the persistence of IgM antibodies for an extended period after primary infection. After the resolution of primary infection, HCMV specific IgM could be detectable for an average of 6 to 9 months [13] [14].

To establish a diagnostic value for the HCMV-pp65 antigenemia occurring in HIV-infected patients, HCMV-pp65 antigen in blood samples was assayed in 90 individuals. Four (4.44%) subjects were found to have positive HCMV-pp65 antigenemia. Several studies have suggested that the HCMV-pp65 antigenemia assay may have useful predictive values for the diagnosis and monitoring of HCMV disease in HIV-infected patients. Consistent with the results, we determined that 3 patients had also positive results for HCMV-IgM, HCMV-pp65, and HCMV-PCR assays which suggested that those subjects have active HCMV viremia.

Regarding the HCMV PCR in the current study, only 3.33% (3/90) of the study subjects were PCR positive for HCMV-DNA. This is consistent with the other reports from Ethiopia which were detected at 4.1% [11] and South Africa, which were reported to be 5.2% [15]. In addition, our findings were relatively lower than findings from Tanzania (22.6%) and Kenya (17%) [16]. With respect to gender, 66.7% of participants with confirmed HCMV DNA were females and this is consistent with the other performed HCMV assays. Several lines of evidence consistently reported prevalence rates that were slightly higher in women than in men. Population-based evaluation of HCMV prevalence that have been derived from the National Health and Nutrition Examination Survey (NHANES), estimated that more female patients (63.5%) than male (54.1%) were infected [17] [18].

Positive viremia along with lower CD4 counts have been reported as a predictor of HCMV diseases and are associated with increased mortality regardless of the plasma HIV RNA [19]. Among HIV/AIDS patients, HCMV may present with a wide range of clinical manifestations and results in a significant exacerbation of morbidity and mortality [19] [20]. Based on several report, HCMV DNA detection among HIV-infected patients in the current study could be an early predictor of disease progression. Another report also supports the hypothesis that HIV infected patients with DNAemia/positive plasma HCMV DNA were shown to be correlated with a 2.5-fold increased risk of death over 650 days [21]. Similarly, another report from South Africa suggested that subclinical HCMV viremia was an independent risk factor for mortality among adult males living with HIV [22]. These findings suggest the need for early diagnosis and subsequent treatment of HCMV infection.

4. Conclusion

In this study, we reported that the molecular prevalence of HCMV DNA among

HIV undergoing ART was 3.33%. HCMV viremia is strongly associated with the development of life-threatening HCMV disease. Therefore, an early routine detection is highly critical in monitoring and preventing the progression of HIV diseases. In addition, the use of anti-HCMV therapy for HCMV viremic individuals parallel to ART is also recommended to reduce the burden of HCMV complications and consecutively extending the life of HIV/AIDS patients. We recommend further investigations with a larger sample size at a national and regional level to draw the overall picture of HCMV-HIV co-infection, which in turn helps to develop national preventive and treatment strategies.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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